UNIVERSITY OF LIVERPOOL

Antigen expression and host-parasite interactions of *Plasmodium falciparum* infections in Malawian paediatric patients

Thesis submitted in accordance with the requirements of the University of Liverpool and College of Medicine, University of Malawi for the degree of

Doctor of Philosophy

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March 2013

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DECLARATION

This thesis is the result of my own work except where indicated. Studies in this thesis were done in conjunction with other studies and in some instances work was shared. My contribution towards the work is as follows:

Activity	**Responsibility**
Sample Processing	Shared
Quantitative real-time PCR	Sole
Platelet-mediated clumping assay	Sole
Platelet mobilisation assay	Sole
Statistical data analysis and presentation	Sole
Thesis preparation	Sole

The material contained in the thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification elsewhere.

Dumizulu L. Tembo

ABSTRACT

Introduction The process of sequestration involving specific cytoadhesion between parasite ligands expressed on the surface of the parasitised red blood cells (pRBC) and host vascular endothelium contributes to pathogenesis of severe falciparum malaria. A major polymorphic surface antigen implicated in cytoadhesion is the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is encoded by the *var* multigene family that is sub-divided into three main groups: A, B and C, according to sequence similarities in coding and non-coding sequences. PfEMP1 has variant adhesive phenotypes, some of which interact with ABO blood groups to form rosettes and some involved in apparent formation of platelet-mediated clumps of infected erythrocytes that are thought to contribute to disease severity. With heavy HIV burden concentrating in areas with high malaria rates, co-infections are common. Both HIV and malaria interact with the host immune system, resulting in a complex activation of immune cells and subsequent dysregulated production of cytokines and antibodies. However, there is limited information on the molecular mechanisms of interaction between the two infections.

Methods Real time PCR was used to: 1) compare abundance of the three main *var* groups and measure the level of cytokine production and receptor expression utilising the resources of a clinicopathological study of 20 Malawian fatal paediatric malaria patients divided into three diagnostic groups: circulating and sequestered parasites (CM1); circulating and sequestered parasites plus perivascular pathology (CM2) and parasitaemic control (PC) groups; and 2) determine the effect of host ABO blood group on expression of *var*/PfEMP1 subtypes mediating platelet-mediating clumping in 65 Malawian paediatric patients with uncomplicated malaria (UM).

Results While there were no significant associations between ABO blood antigen groups with the clumping phenotype in UM patients, an abundance of *var* upsA and C transcripts were expressed in CM2 and the PC ($p \leq 0.001$) groups. However, a very different expression pattern was observed in the CM1 group, which were mostly HIV positive (80%), with *var* gene group upsB being more abundant than in the other two diagnostic groups ($p \leq 0.001$). This result was supported by different cytokine/receptor upregulation between HIV positive and HIV negative children, with significant upregulation of TNF in HIV negative children ($p \leq 0.05$).

Conclusions This data suggests that perivascular pathogenesis in naturally infected children is associated with differential *var* gene expression in the body. HIV disruption of cytokine release affects receptor regulation and influences parasite antigen expression.

ACKNOWLEDGEMENTS

I would like to thank Malawi-Liverpool-Wellcome Trust for giving me one of their in-house scholarships that allowed me to do this PhD programme. My heartfelt thanks to my supervisors for their guidance, support and assistance throughout my PhD; Dr Jacqui Montgomery, who pretty much taught me everything I know about molecular biology and *var* genes, Prof Alister Craig whose every "what do you mean?" made me really think deep about the science and Dr Wilson Mandala who help me a lot with all the admin work, making me always one step ahead. A special thank you to my advisors: Prof. Victor Mwapasa who gave me the best advice ever, "Relax" and it worked (most of the time); Dr Samuel Wassmer who makes all things sciency very easy and for helping me out with all the clump techniques. I also would like to thank Prof. Malcolm Molyneux for gently convincing me to pursue this PhD programme.

I would like to thank members of the Blantyre Malaria Project starting with Dr Karl Seydel for supplying with parasite lines and helping me whip this thesis into shape. Thank you to Dr Rabia Mukadam for sparing me some space in the ICEMR lab and Godfrey Mvula who taught me everything I know about parasite culturing. Thank you to Nurse Grace Matimati who performed a wondrous miracle of getting me all samples I needed in record time. I also would like to thank all study participants and their guardians.

To my family, mum, Fushie, Khadijah, Atia and Mimi who had to put with my whinging and whining and moodiness and whatever else they found insufferable about me, you know you love me (zizipizga we nganya- bear with me) and I love you too and Dad I know you are proud.

Lastly but not at all in the least, to my first love, God, my father in heaven, the author of my life who is able to do exceedingly, abundantly so much more that I can ever dream or hope for, to Him be everlasting glory forever and ever Amen!

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LIST OF ABBREVIATIONS

ACT	Artemisinin Combination Therapy
ADH	Adenosine Diphosphate
AIDS	Acquired Immunodeficiency Syndrome
AM	Asymptomatic Malaria
ANC	Antenatal care
AMA1	Apical Membrane Protein 1
ANOVA	Analysis of Variance
ATS	Acidic Terminal Segment
ART	Anti-retroviral treatment
BBB	Blood brain barrier
bp	Base pair
cDNA	Copy Deoxyribonucleic acid
CIDR	Cysteine-rich Inter-domain Region
CM	Cerebral Malaria
CD	Cluster of Differentiation
CD4	Defines MHC class II restricted T cell subsets
COM	College of Medicine, University of Malawi
CSA	Chondrotin Sulphate A
CSP	Circumsporozoite Protein

DBL	Duffy Binding Ligand
DC	Dendritic Cell
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphates
EBA 175	Erythrocyte-binding Antigen 175
EC	Endothelial Cell
EDTA	Ethylenediaminetetraacetic acid
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GLURP	Glutamate-rich Protein
gDNA	Genomic Deoxyribonucleic acid
GPBB	Glycoprotein Ib/beta Polypeptide
GPI	Glucose-G-Phosphate Isomerase
HA	Hyaluronic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Inter-cellular Adhesion Molecule 1
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M

IL	Interleukin
IPT	Intermittent preventive treatment
IRS	Indoor residual spraying
ITNS	Insecticide-treated bednets
LDH	Lactate Dehydrogenase
LLITN	Long lasting insecticide treated nets
MDM	Monocyte-Derived Macrophage
MIN	Minute
MIS	Malaria Indicator Survey
MLW	Malawi-Liverpool wellcome Trust Clinical Research Programme
MM	Mild Malaria
MSP	Merozoite Surface Protein
NaCl	Sodium Chloride
NCAM	Neural Cell Adhesion Molecule
NMCP	National Malaria Control Programme
NO	Nitric Oxide
NTS	Non-typhoid Salmonellae
PAM	Pregnancy Associated Malaria
PAT	Platelet Aggregation Test
PB	Peripheral Blood

PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PC	Parasitic Control
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PECAM-1	Platelet Endothelial Cell Adhesion Molecule 1
PfEMP1	*Plasmodium falciparum* Erythrocyte Membrane Protein-1
Pfmc-2TM	*Plasmodium falciparum* Maurer's Cleft-2 Transmembrane
6PGD	6-Phosphogluconate Dehydrogenase
PMI	President's Malaria Initiative
PPP	Platelet-Poor Plasma
PRP	Platelet-Rich Plasma
pRBC	Parasitised Red Blood Cell
QECH	Queen Elizabeth Central Hospital
RBC	Red Blood Cell
RESA	Ring-infected Erythrocyte Surface Antigen
RIF	Repetitive Interspersed Family (also RIFIN?)
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT	Room Temperature

RT-qPCR	Real-time Quantitative Polymerase Chain Reaction
SP	Sulfadoxine-Pyrimethamine
SEC	Second
SERA5	Serine Repeat Antigen 5
SM	Severe Malaria
SNP	Single Nucleotide Polymorphism
SRD	Severe Respiratory Distress
STEVOR	Subtelomeric Variable Open Reading Frame
UM	Uncomplicated Malaria
TFCR	Transferrin Receptor
TM	Transmembrane
TNF	Tumour Necrosis Factor
TNFR	Tumour Necrosis Factor Receptor
VCAM-1	Vascular Cell Adhesion Molecule 1
VSA	Variant Surface Antigen
vWF	von Willebrand Factor

PUBLICATIONS

1. Tembo, D.L., Montgomery, J., Craig, A.G., Wassmer, S.C. (2013) **A Simple Protocol for Platelet-mediated Clumping of Plasmodium falciparum-infected Erythrocytes in a Resource Poor Setting**. J. Vis. Exp. (75): 1-6

2. Martincová E, Voleman L, Najdrová V, De Napoli M, Eshar S, Gualdron M, Hopp CS, Sanin DE, Tembo DL, Van Tyne D, Walker D, Marcinčiková M, Tachezy J, Doležal P. **Live Imaging of Mitosomes and Hydrogenosomes by HaloTag Technology**. PLoS One 7(4) 2012

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Chapter 1

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Global Malaria Burden

Malaria is a disease that has plagued the human race for centuries. The word malaria is derived from two medieval Italian words “mal” and “aria” which mean “bad air”. It was believed to be caused from ghastly smells from swamps in the 14th century. Malaria is a vector-borne infectious disease caused by a eukaryotic protozoan of the genus *Plasmodium*. It is widely spread in the tropics and subtropical regions, which include parts of the Americas, Asia, and Africa. 70% of malaria clinical attacks occur in sub-Saharan Africa and 25% are in highly populated South East Asia (Snow, Guerra et al. 2005). 90% of the mortality from malaria occurs in Africa with poverty contributing to the high mortality.

Malaria is regarded as one of the most important parasitic infections worldwide with approximately 40% of the world’s population being affected by the disease once or more in their lifetime. Despite control measures aimed at decreasing disease burden, there has been a tremendous increase in the annual number of cases from 221 million in 2002 (Snow, Guerra et al. 2005) to approximately 500 million cases and with about one million fatalities annually in sub-Saharan Africa alone (Hay, Guerra et al. 2009). In 2007, an estimate of 2.4 billion people were at risk of malaria with 60% of this population living in areas designated as having stable *P. falciparum* transmission; Central and South East Asia (CSE Asia) and Africa (Hay, Guerra et al. 2009). In 2009, a study carried out in 81 of the most malaria prone countries with the aim of

estimating global *Plasmodium falciparum* infection (Hay, Guerra et al. 2009) found that of the 1.38 billion people at risk of contracting *P. falciparum* malaria, the majority were found in CSE Asia, including Yemen and Saudi Arabia (0.69 billion) and Africa (0.66 billion), with the remainder in Latin America. Recently, the population at risk of *P. falciparum* malaria is estimated to be between 1.3-1.44 billion (Gething, Patil et al. 2011). According to the 2011 World Malaria Report, malaria mortality rates have fallen by more than 25% globally since 2000 and by 33% in Africa through concerted malaria control programmes.

Malaria is geographically restricted to certain regions of the world and remains well established in areas with suitable transmission climates. There are three main climate factors that promote malaria: temperature, precipitation and humidity (Pampana 1969). Temperature affects many parts of the malaria cycle; temperatures of about 27˚C are favourable for the extrinsic cycle of the parasite. Higher temperatures also increase the number of blood meals which results in increased egg production (Martens, Niessen et al. 1995). Rainfall has proportional influence on humidity and temperature. Different *Anopheles* species prefer different water bodies; some prefer shallow collections of fresh water such as puddles whereas others prefer salty or brackish water. The mosquito lifespan is long and transmission intensifies during and just after the rainy season (Martens, Niessen et al. 1995). Heavy rainfall washes away mosquito larvae.

Urbanisation has also been identified as a potential factor influencing malaria transmission. For example, *An. gambiae,* which prefers to breed in clean water, is found breeding in polluted water in urban areas due to the mosquitoes' ability to evolve and adapt to

natural or man-made environments (Keating, Macintyre et al. 2004; Sattler, Mtasiwa et al. 2005; Klinkenberg, McCall et al. 2008). These mosquitoes have a local adaptation or phenotypic plasticity in these urban populations, resulting into an increase in malaria incidences in high density urban population (Donnelly, McCall et al. 2005; Klinkenberg, McCall et al. 2005). Thus, in additional to developing resistance to the insecticides used in the ITNs *An. gambiae ss* might adapt to the urban environment behaviourally which could comprise other control methods.

The degree of *P. falciparum* endemicity also varies depending upon geographical location and socio-economic factors (Figure 1.1). In areas of high infection like the sub-Sahara, the greatest suffering is borne by children less than 5 years old and by pregnant women who together contribute approximately 3,000 deaths per day. By contrast, in areas of low transmission, all age groups are at equal but reduced risk of infection (WHO 2011). In 2002, as high as 70% of all clinical events attributable to *P. falciparum* were concentrated in the African region compared to only 25% in South East Asia (Snow, Guerra et al. 2005). It has been estimated that the annual incidence of severe malaria was 10.7 million in Africa and only about a third of that in Asia and the Americas (Hay, Guerra et al. 2009). Such high prevalence of the disease in Africa has led to malaria being ranked as one of the major killers alongside HIV/AIDS.

The distribution of malaria in endemic countries is further sub-divided according to the degree of endemicity. Areas in which the infection prevalence is less than 10% are said to be hypoendemic, and those where the infection prevalence is between 11-50% are referred to as mesoendemic. Regions are described as holoendemic and hyperendemic if the prevalence is above 50% (Hay, Guerra et al. 2009).

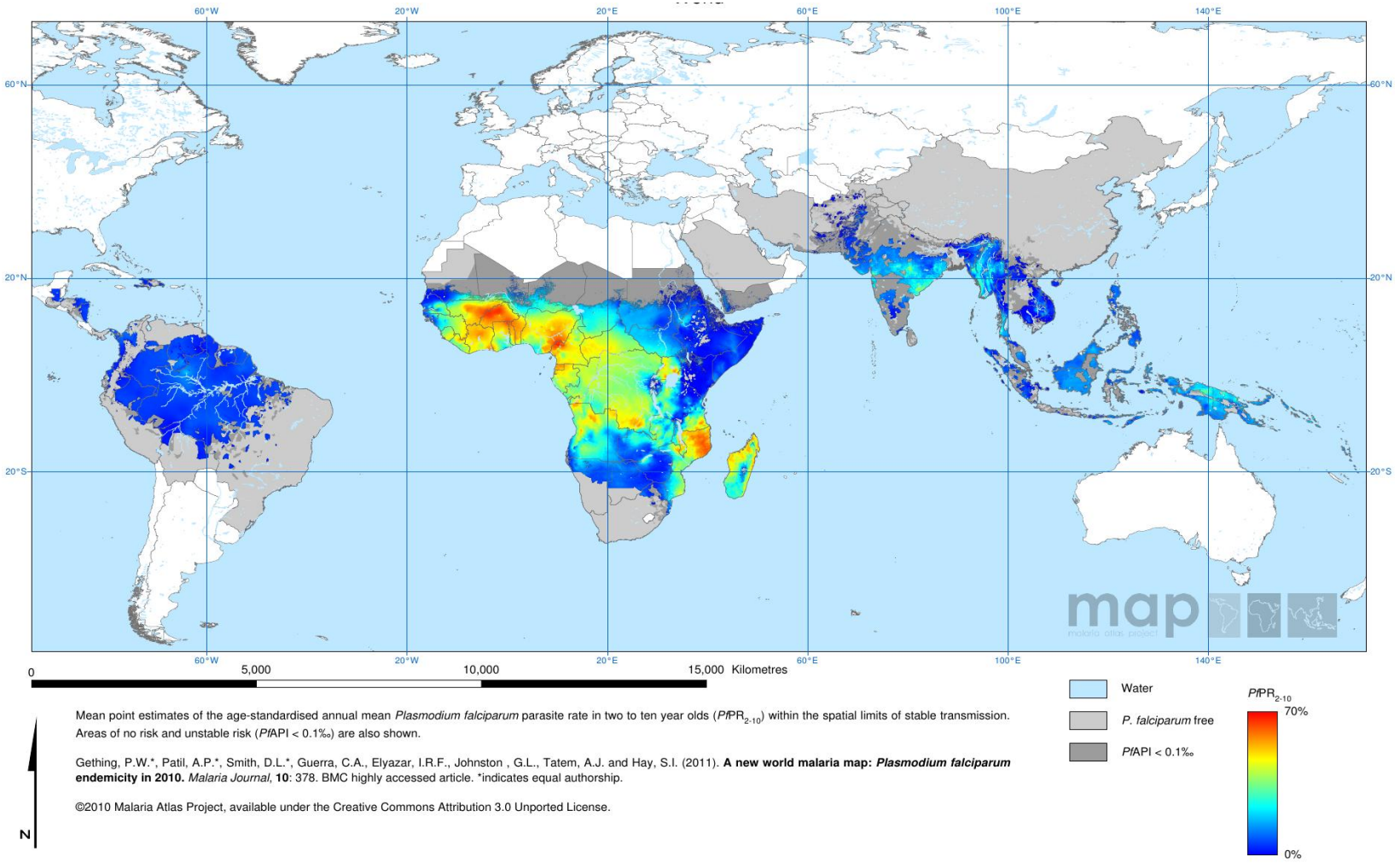


Figure 1.1. The 2010 Spatial Distribution of *P. falciparum* malaria by endemicity

(From Gething et al. Malar J. 2011: 10 (378))

1.2 Malaria transmission

Malaria is caused by protozoa of the genus *Plasmodium,* family Plasmodiidae. There are over 120 species of *Plasmodium* that can infect mammals, reptiles and birds. Human malaria is transmitted by female anopheline mosquitoes during a blood meal, a source of protein for egg development and maturation. Not all *Anopheles* species transmit malaria, and only about 30-40 different *Anopheles* mosquitoes are of local clinical importance. The major malaria vectors in sub-Saharan African are *An. gambiae s.s*, *An. arabiensis* and *An. funestus* (Gillies 1968). Currently, there is no vaccine available for malaria, therefore vector control is one of the most important methods for malaria prevention. However, over the years the main vectors have evolved resistance to commonly used insecticides, increasing their chances of survival such that control of these vectors is a challenge (Greenwood and Mutabingwa 2002).

There are four *Plasmodium* species that cause human malaria: *P. falciparum, P. vivax, P. ovale* (which is actually two species), and *P. malariae;* the latter can also cause disease in primates. Recently, *Plasmodium knowlesi*, a primate malaria pathogen has also been identified to cause clinical malaria in humans (Singh, Kim Sung et al. 2004). The most virulent disease is caused by *P. falciparum* which contributes greatly to child mortality annually. The special disease presentation of this species, compared to other *Plasmodium* that infect humans, has been attributed to the ability of *P. falciparum* to cause organ specific diseases such cerebral malaria and placental malaria (Pongponratn, Riganti et al. 1991; Berendt, Tumer et al. 1994; Turner, Morrison et al. 1994).

1.3 Malaria transmission and control in Malawi

1.3.1 Transmission

In Malawi, malaria is endemic and continues to be a major public health problem, with no reduction in disease incidence observed over the past decade despite a change of first-line anti-malarial treatment in 2007 and intensification of vector control programmes (Roca-Feltrer, Kwizombe et al. 2012). There has been a gradual increase in the number of outpatient visits between 2001 and 2010 (Figure 1.2).

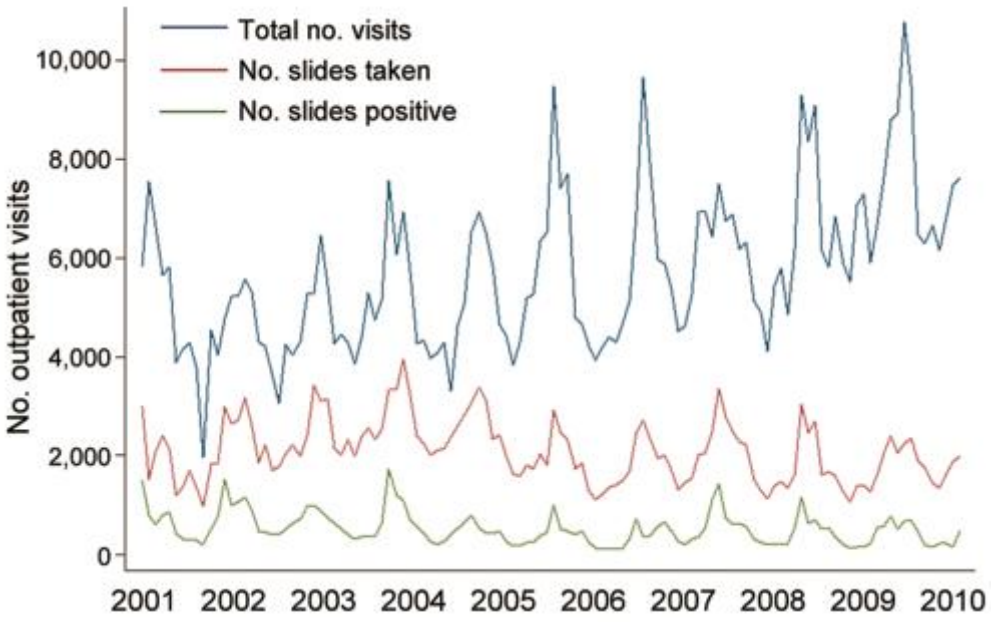
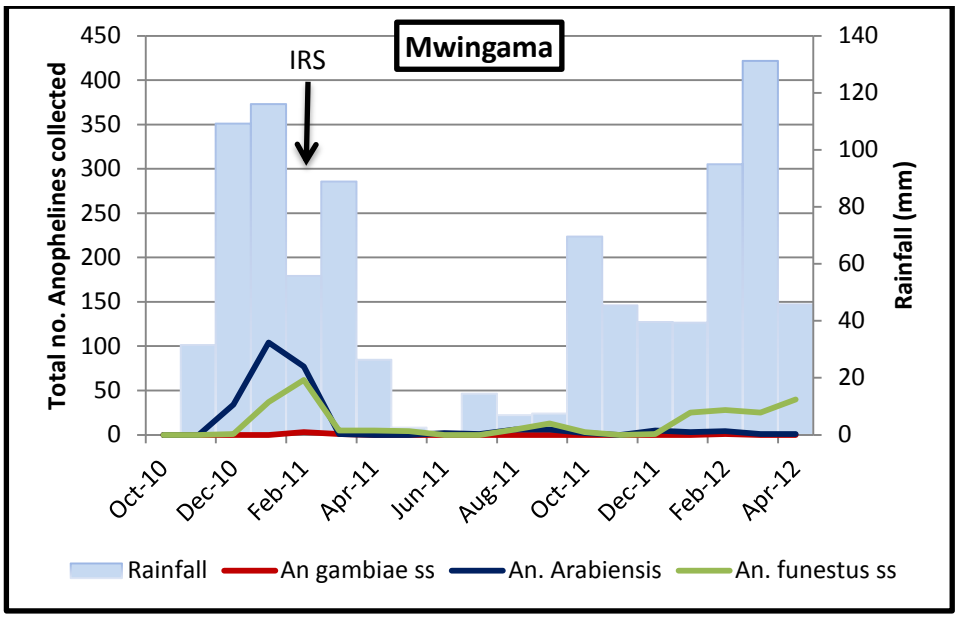


Figure 1.2. Temporal trends of total monthly outpatient visits, malaria slides taken, and parasitaemia-positive slides. Data was recorded from the Pediatric Accident and Emergency Unit at Queen Elizabeth Central Hospital, Blantyre, Malawi, 2001–2010 (*From Roca-Feltrer et al. Emerg Infect Dis 18(2) 2012 272-8)*

An estimated 6 million cases occur annually with 98% of malaria infections caused by *P. falciparum* and transmitted by *An. funestus*, *An. gambiae,* and *An. arabiensis* (PMI 2012). About 40% of all hospitalisation of children less than five years old and 34% of all outpatient visits across all ages are due to malaria (PMI 2012). Nearly 60% of all hospital deaths in children less than five years old are attributed to malaria and anaemia. There are several potential independent etiological factors associated with severe anaemia in Malawian children; bacteremia, malaria, hookworm, HIV infection, vitamins A and B_{12} deficiency (Calis, Phiri et al. 2008). The underlying cause was identified as failure of red cell production (Boele van Hensbroek, Calis et al. 2010).

Transmission rates are intense all year round, especially during the rainy season between November- April for areas lying within metres of sea level. In other areas, transmission lessens during the dry season (October to November) and peaks again in the rainy season. Highland regions (>1500m) of Africa historically have been considered free of malaria, until the late 1980s when concern arose that high elevation malaria transmission may be increasing (Lepers, Deloron et al. 1988; Some 1994; Malakooti, Biomndo et al. 1998; Lindblade, Walker et al. 1999). Hypotheses about the reasons for this include changes in climate, land use and demographic patterns (Bouma, Baeza et al. 2011; Chaves, Hashizume et al. 2012; Ermert, Fink et al. 2012).

Figure 1.3 shows an example of Anopheles distribution in Tsekera area which is close to the river, has a lot of water pools and hosts a lot of livestock compared to Mwingama, an area further from the river, has less water pools and livestock, both found in Chikwawa district.



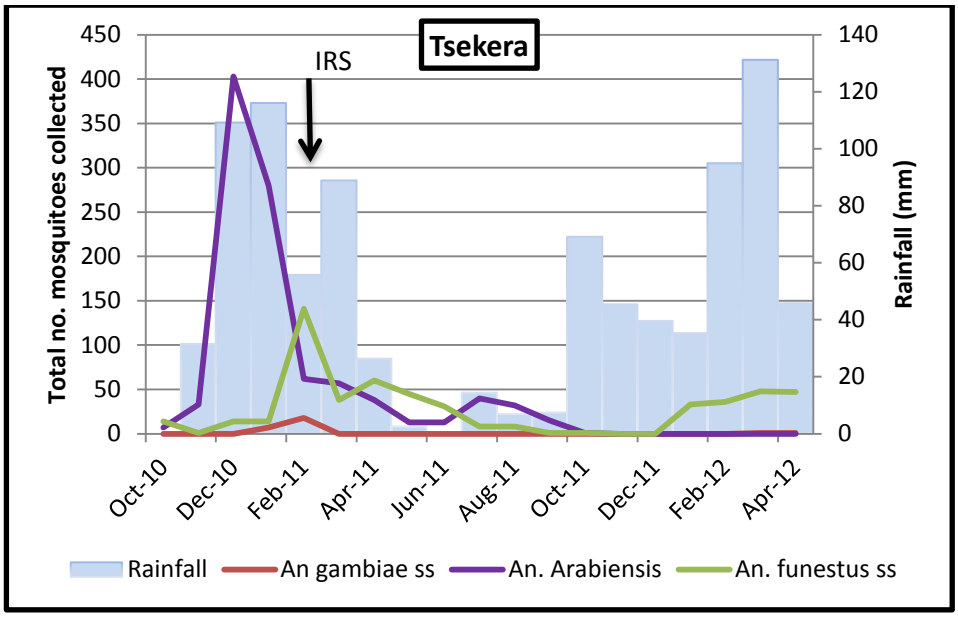


Figure 1.3. *Anopheles* mosquito species abundance in two sentinel sites and monthly rainfall distribution for Chikwawa district. While annual rainfall pattern is constant throughout the district, there has been a significant reduction of all Anopheles mosquitoes since the introduction of IRS (*Benjamin Nyoni, Unpublished data*).

1.3.2 Control Interventions

Malawi has adopted two major malaria control interventions: treatment and vector control.

1.3.2.1 Chemoprophylaxis and Chemotherapy

1.3.2.1.1 Medication and treatment

For several years, African countries have used drugs comprising of quinoline and antifolate types to treat malaria. In Malawi, chloroquine was used as a first-line drug to treat *P. falciparum* infections until world-wide *in vivo* resistance was detected. In 1993 Malawi switched to sulfadoxine-pyrimethamine (SP) as first-line treatment and quinine as the second-line drug to treat malaria, depending on disease severity. While the latter remains efficacious and is still used to treat severe cases, the parasite has long developed resistance for chloroquine and SP, leading to an increase in malaria-related mortality (Trape, Pison et al. 1998; Korenromp, Williams et al. 2003). However, recent research suggests that chloroquine may again be effective following the decline in prevalence of chloroquine-resistant *P. falciparum* in Malawi by 2001 (Laufer, Thesing et al. 2006).

In 2007, Malawi adopted artemisinin-based combination therapy (ACT) as the first-line therapy for fighting *P. falciparum* infections as recommended by WHO for endemic countries. By 2009, another 42 malaria endemic African countries also

changed their treatment regime to ACT and 20 countries adopted the use of rapid diagnostic tests (RDTs) for all-age groups (WHO 2009).

1.3.2.1.2 Intermittent Preventative Treatment (IPT)

In areas of high malaria transmission, the population most at risk comprises pregnant women and children less than five years of age (Greenwood, Fidock et al. 2008). In 1998, WHO recommended the use of IPT which involves administering a predetermined curative dose of antimalarials at specific intervals to individuals in endemic areas before they are parasitaemic (Garner and Gulmezoglu 2006; Briand, Cottrell et al. 2007) in order to control for gestational malaria in pregnant women.

The importance of IPT usage in Malawi was confirmed by clinical trials which reported reduced incidence of gestational malaria, anaemia and low birth weight in pregnant women and their babies (Luxemburger, Ricci et al. 1997; Nosten, McGready et al. 2007), and reduced rates of hospital re-admittance due to malaria and/or severe anaemia in children (Phiri, Esan et al. 2012).

1.3.2.2 Vector control

Vector control is regarded as one of the most successful methods to reduce malaria transmission at the community level. The two most effective vector control methods that Malawi has adopted are:

1.3.2.2.1 Insecticide treated nets (ITNs) and long lasting insecticide treated nets (LLIN)

ITNs decrease malaria transmission by mass killing of *Anopheline* vectors (Takken 2002). ITNs provide 17% protective efficacy compared to no nets and save about 5.5 children each year for every 1000 children protected with ITNs (Lengeler 2004). ITNs reduced the incidence of uncomplicated malarial episodes of *P. falciparum* by 50% and 62% compared to no nets in areas with stable malaria and areas with unstable malaria, respectively (Lengeler 2006). Recently, WHO has recommended LLIN which have insecticide incorporated into the net fibres rather than soaked into the net at regular intervals as for ITNs (Norris and Norris 2011).

In Malawi, the current National Malaria Control Programme (NMCP) supported a three-pronged approach to ITN distribution: 1) routine distribution of free pyrethroid-treated LLINs through antenatal care and expanded programmes in immunisation clinics, 2) periodic mass campaigns covering the entire population, and 3) traditional social marketing through private sector outlets. NMCP hopes to achieve universal coverage with ITNs which is defined as one net per two people (PMI 2012). According to the 2010 Malaria Indicator Survey (MIS), nearly 60% of households in Malawi owned one or more ITNs and that 55% of children less than five and 49% of pregnant women slept under an ITN the previous night (PMI 2012).

1.3.2.2.2 Indoor spraying with residual insecticides

The use of indoor residual spraying (IRS) to reduce malaria has been adopted since the 1940s and 1950s (De Mellion 1936; Russell 1955.; Alilio, Bygbjerg et al. 2004)

and it is now widely used as one of the primary interventions of malaria control as recommended by WHO.

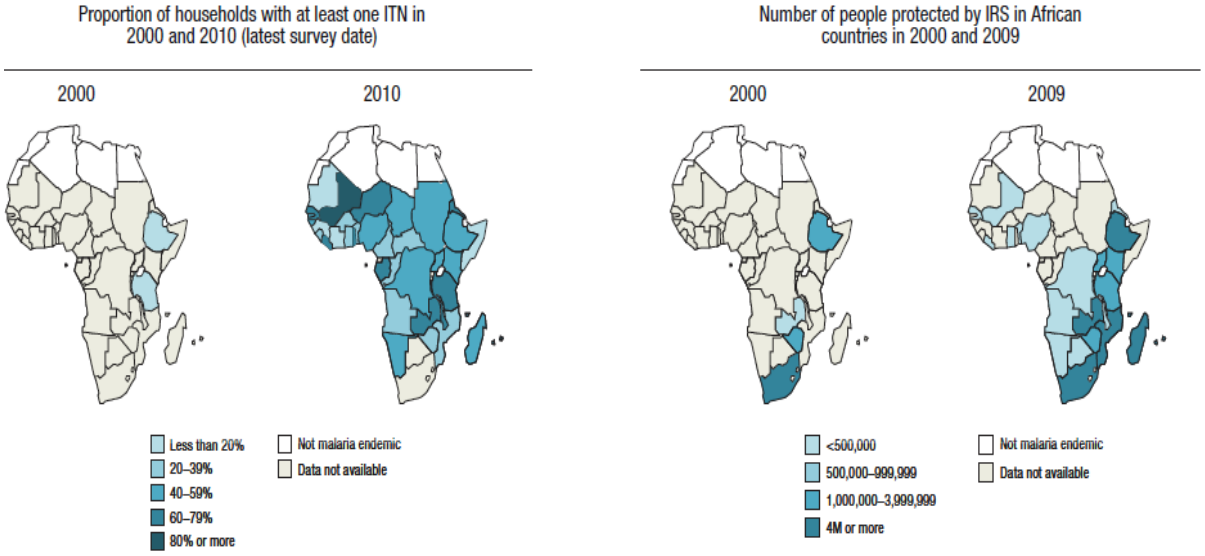


Figure 1.4. Progress in vector control coverage in sub-Saharan Africa from 2000 to 2010 with adjustments from Global Malaria Programme (*WHO 2011*).

Pyrethroid IRS is included in the Malawi Malaria Strategic Plan of 2011-2015 as a key malaria prevention strategy (PMI 2012). In the years 2007-2009, the President's Malaria Initiative (PMI) supported two initial spraying rounds covering 27,000 houses in the Nkhotakota district. Based on the success of these efforts (Skarbinski 2012), the Malawi Ministry of Health expanded IRS to a total of 7 districts: Chikhwawa, Karonga, Salima, Mangochi, Nkhotakota, Nsanje and Salima, covering 500,000 houses and protecting an estimated 2.5 million people (PMI 2012).

However, the Cinderella story does not seem to last as the first evidence of insecticide resistance in *An. funestus* were reported on one of the islands in Lake Malawi (Hunt, Edwardes et al. 2010). Other cases of possible pyrethroid resistance in *An. funestus* have been reported in some areas where IRS has been implemented (PMI 2010). In spite of this, NMCP is planning to intensify IRS to 12 high malaria burden districts by 2015.

1.3.2.2.3 Larvicides

The future in malaria control is now heading towards integrated vector management, targeting both larval and adult mosquitoes (WHO 2003; Townson H 2005; WHO 2007). While ITN and IRS use is currently the priority, there is growing interest in arresting aquatic stages of malaria vectors with microbial larvicides (Shililu, Maier et al. 1998; Killeen, Fillinger et al. 2002; Killeen, Fillinger et al. 2002; Utzinger, Tanner et al. 2002; Fillinger, Sonye et al. 2004; Fillinger and Lindsay 2006; Gu, Utzinger et al. 2008). So far, microbial larvicides are only being used in Tanzania (Geissbuhler, Chaki et al. 2007; Fillinger, Kannady et al. 2008). However, there are plans to expand larvicide usage to 17 countries included in the PMI.

Malawi is in the process of investigating options for larviciding in 10 districts along the lakeshore and Shire Valley areas. Seven out of the 10 earmarked districts for larviciding are currently designated as IRS districts.

1.4 THE PLASMODIUM FALCIPARUM PARASITE

1.4.1 *P. falciparum* life cycle

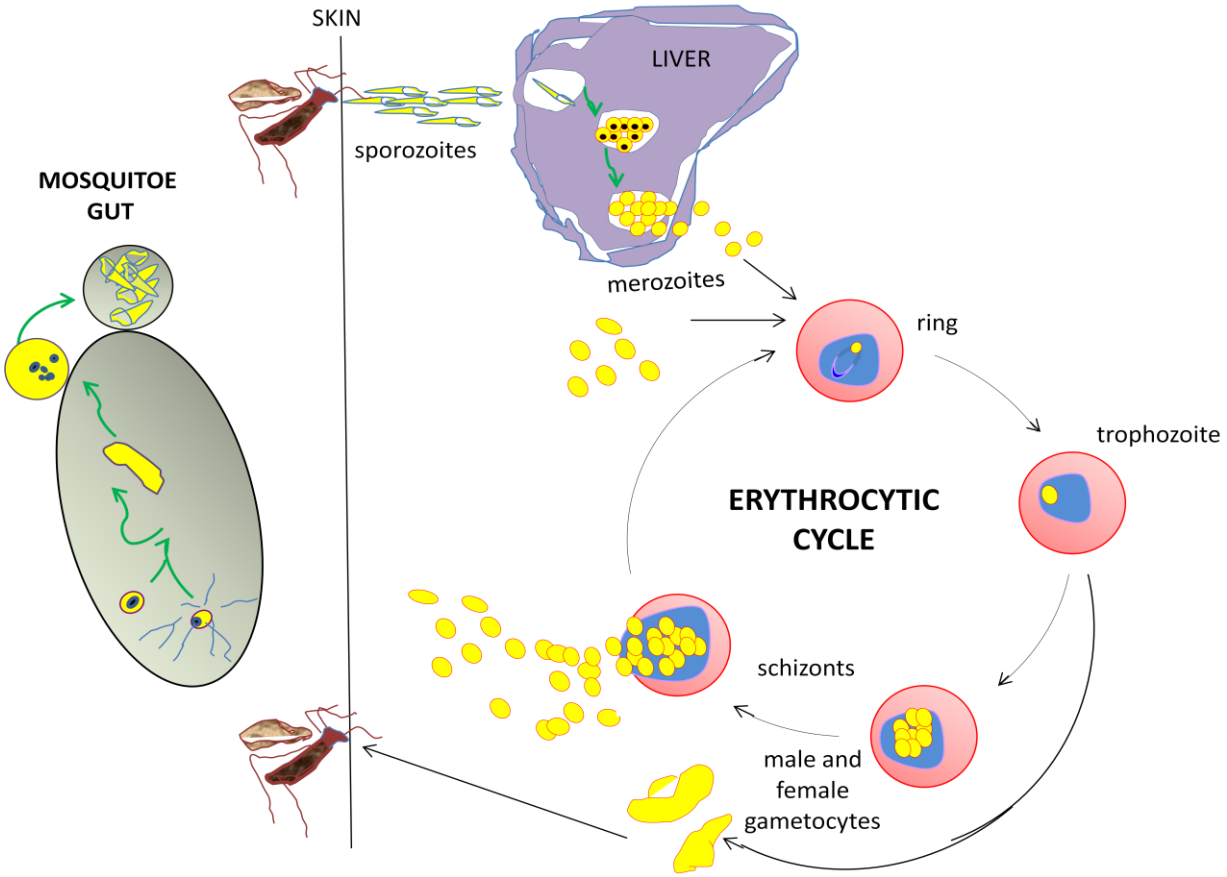


Figure 1.5. *P. falciparum* life cycle.

P. falciparum has a complicated life cycle with asexual reproduction occurring in the mammalian host and sexual reproduction in the anopheline mosquito vector. Host infection commences upon the dermal inoculation of sporozoites by an infected female mosquito. The sporozoites circulate for up to 45 minutes before invading liver hepatocytes where they undergo asexual reproduction for approximately one week to

form large intracellular hepatic schizonts containing thousands of merozoites. *P. vivax* and *P. ovale* infections can lie dormant in the liver for weeks or months before reactivating and forming a hepatic schizont. The entire pre-eryhrocytic phase lasts about 5–16 days depending on the parasite species: an average 5-6 days for *P. falciparum*, 8 days for *P. vivax,* 9 days for *P. ovale,* and 13 days for *P. malariae* and 8-9 days for *P. knowlesi.*

The pathogenic process begins when the swollen hepatocytes eventually rupture, discharging merozoites into the bloodstream where they rapidly invade erythrocytes. The merozoite recognises, attaches and then enters RBCs by multiple receptor–ligand interactions in as little as 60 seconds. This quick invasion minimises exposure of the parasite surface antigens from the host immune response. *P. vivax* invades only Duffy blood group-positive RBC, using the Duffy-binding protein and the reticulocyte homology protein, expressed mostly on the reticulocytes. On the other hand, *P. falciparum* uses several different receptor families and alternate invasion pathways that are highly redundant. The merozoite has special apical secretory organelles: micronemes, rhoptries, and dense granules that help the invasion process. The merozoite attaches to the surface of the RBC forming a stable parasite–host cell junction. Following this, the parasite pushes its way through the erythrocyte bilayer and creates a parasitophorous vacuole to seal itself from the host cell cytoplasm.

The parasite develops within a membrane-bound parasitophorous vacuole first as a trophozoite and then, during multiple nuclear divisions known as schizogony, as a

schizont. When the schizont matures, it ruptures the host erythrocyte, releasing merozoites that rapidly invade fresh erythrocytes in circulation, thereby continuing the asexual life cycle. A small proportion of merozoites develop into sexual forms (male and female gametocytes). The gametocytes starts as a round body measuring 2 µm across and then mature into twin crescent shapes found in the peripheral blood or in the spleen. The gametocytes enter the peripheral blood approximately 8 to 11 days after the initial infection (Garnham, 1966). Crescents can persist for up to 3 months or even longer. Initially, females outnumber the males 3:1 but towards the end of the infection their numbers become more or less equal (Baker 2010; Kuehn and Pradel 2010). The mature male gametocyte or microgamete measures 9-11 µm in length, and the female gametocyte or macrogametes measuring 12-14 µm in length, are taken into the mosquito gut with a blood meal.

Sexual reproduction occurs in the mosquito, leading to new genetic combinations, before inoculation into new hosts. Inside the gut of the mosquito, the gametocytes undergo sporogony where the gametes fuse to form a zygote, which then undergo meiosis within 12 – 18 hrs after the blood meal to form first an ookinete and five days later develops into an oocyst in the gut wall. The oocyst rapidly divides and bursts, releasing large numbers of sporozoites that migrate to the salivary glands of the mosquito to await injection into a human host during the next blood meal.

1.5. *Plasmodium falciparum* disease and clinical features in African children

The impact of *P. falciparum* infection in endemic African countries is highlighted by the socio-economic burden these countries experience. Children less than five years of age, which this thesis will concentrate on, and pregnant women bear the brunt of the infection although all age groups can be infected. The high disease burden in malaria endemic areas results in individuals acquiring malaria-specific antibodies with age. These antibodies are able to recognise and thus protect individuals against a range of parasite isolate, potentially including those that cause severe forms of disease. Parasites are able to sequester in the placenta of pregnant women causing what is referred to as placental malaria (PM) (Steketee, Nahlen et al. 2001; Guyatt and Snow 2004). PM is risky to both the mother and child as it can lead to severe malarial anemia in the mother and low birth weight for the baby (Murphy and Breman 2001). PM is more common in women who are pregnant for the first time (primigravid) than those who have been pregnant before (multigravid) as the primigravida lack antibodies to placental binding parasites (Fried, Nosten et al. 1998; Beeson, Brown et al. 1999; Beeson and Rogerson 2004).

Malaria is classified into three categories: asymptomatic malaria (AM) mild or uncomplicated malaria (UM) and severe malaria (SM). UM is mainly characterised by persistence, but not worsening, of the initial symptoms such as fever that are observed approximately 8 days after the introduction of the parasite in the human host. These symptoms are suggested to be due to the release of parasite toxins during erythrocytic rupture (Bates, Berendt et al. 1992; Berendt, McDowall et al. 1992).

The causes of complicated malaria, commonly known as SM, are complex and not yet fully understood. Severe falciparum malaria can cause acute febrile illness characterised by fever, chills, headache, severe anaemia and splenomegaly, which usually respond promptly to appropriate anti-malarial therapy. SM is sub-divided into three clinical syndromes: cerebral malaria (CM), severe malarial anaemia (SMA) and severe respiratory distress (SRD) (Marsh et al., 1995). Left untreated, the patient will either die in the acute attack or survive but with minimal development of immunity against the next infection, in the case of a first attack. Substantial immunity only develops after an individual has been exposed to malarial antigen in multiple episodes over a number of years (Ho and White 1999). CM has been associated with sequestration of pRBC in brain microvasculature which leads to the blocking of cerebral post-capillary venules that can eventually lead to rupture and haemorrhage. Other mechanisms attributed to CM are cytokine induction of secondary mediator production such as nitric oxide (NO), which is thought to cause decrease of intracranial pressure due to vasodilation (Clark and Rockett 1994). Tumor necrosis factor (TNF), one of the pro-inflammatory cytokines, has been implicated in CM as it causes up-regulation of adhesion receptors as well as modulating effects of NO.

Approximately 5-10 million infected individuals per year develop complications during the acute infection. These complications are manifested differently between adults and children (Table 1.1), with either coma, with metabolic acidosis and hypoglycemia in SRD, as SMA or as renal failure and pulmonary edema in adults (White and Ho 1992; Bejon, Berkley et al. 2007). Disease progression is influenced by both host

and parasite factors. Various cellular and molecular strategies allow the parasite to evade the human immune response (White and Breman 1998). In African children, the commonest presenting syndromes associated with mortality are CM; recurrent convulsions; metabolic dysfunction, which manifests itself as hypoglycaemia or lactic acidosis; or symptomatic anaemia (Marsh, Forster et al. 1995; Newton and Krishna 1998). The overall case fatality rate from severe falciparum malaria varies from 15 to 20%, with the highest mortality resulting from CM with coma or respiratory distress (Newton and Krishna 1998; Newton, Hien et al. 2000).

Table 1.1. Differences in clinical presentation of falciparum malaria infections between adults and children in Africa

Signs/Symptoms	Adult	Children
Anaemia	Common	Very common
Convulsions	Common	Very common
Pre-treatment hypoglycaemia	Less common	Common
Metabolic acidosis	Less common	Common
History of cough	Uncommon	Common
Jaundice	Very common	Common
Renal failure	Common	Less common
Pulmonary oedema	Less common	Rare
Duration of illness	5 – 7 days	1 – 2 days
Resolution of coma	2 – 4 days	1 – 2 days

Malaria retinopathy is a diagnostic tool for CM using a set of five retinal abnormalities: retinal whitening, discolouration of the retinal vessels to orange or white, haemorrhages and papilledema (reviewed in (Lewallen, Harding et al. 1999; Beare, Taylor et al. 2006)) that are unique to SM and common in children with cerebral malaria, and have been linked to risk of death and length of coma. Retinopathy has been shown to be 95% sensitive and shows specificity of 90%. In agreement with other studies (Taylor, Fu et al. 2004; White, Lewallen et al. 2009) and in support of mathematical models and immunological evidence (Gupta, Maiden et al. 1996; Bull, Lowe et al. 1998; Craig and Scherf 2001; Kyes, Horrocks et al. 2001; Ofori, Dodoo et al. 2002; Peters, Fowler et al. 2002; Lavstsen, Magistrado et al. 2005), Malawian children with CM confirmed by retinopathy show less genetically mixed parasites compared to retinopathy negative children (Milner, Valim et al. 2012).

1.6 Parasite population and genotypes

It is necessary to study parasite populations in order to better understand the factors that have an impact on the development of immunity in different endemic areas. In the 1960s, it was suggested that many people in malaria endemic areas carry genetically variant parasites in an active infection (Wilson, McGregor et al. 1969). Proof of heterogenous parasite populations was later provided through enzyme typing, by which variations in three parasite enzymes: glucose-6-phosphate isomerase (G6PI), lactate dehydrogenase (LDH) and 6-phosphogluconate dehydrogenase (6PGD) were found in the peripheral blood samples of *P. falciparum*-infected women and children.

Each enzyme variant represented a different allele of a single gene (Carter and McGregor 1973). There is now clear evidence that an individual can carry a mixture of parasites of different genetic background (Kun, Missinou et al. 2002). The challenge lies in the identification of these genotypes as some parasite subpopulations may "hide" from the circulation by sequestering in the different organs of the host during latter stages of their asexual cycle. Research in this area has taken a new turn in trying to uncover potential competition between genotypically different clones in the human host, in which the situation is complicated when parasites are faced with treatment and the host immune system.

The most common and simple method used for parasite genotyping is performed using three single-copy genes, the glutamate rich protein (*glurp*) gene, located on chromosome 10 (Borre, Dziegiel et al. 1991) and the merozoite surface membrane proteins 1 and 2 (*msp*1 and *msp*2) located on chromosome 9 (Tanabe, Mackay et al. 1987) and 2 (Smythe, Peterson et al. 1990), respectively. These highly polymorphic loci are convenient typing markers (Felger, Tavul et al. 1994; Contamin, Fandeur et al. 1995; Ntoumi, Contamin et al. 1995; Ntoumi, Rogier et al. 1997) because they possess numerous alleles. The *glurp* alleles are identified by size polymorphism of one domain, due to variable copy number of sequence repeats (Borre, Dziegiel et al. 1991). The various *msp*1 alleles are grouped into three families: K1, MAD20 and RO33 according to their reference isolates, based on variable nucleotide sequence and copy number of repeats in their block 2 region (Tanabe, Mackay et al. 1987; Zwetyenga, Rogier et al. 1998). *msp*2 has two allelic families, 3D7 and FC27, which differ in

nucleotide sequence and copy number of repeats of the central domain of the gene (Smythe, Peterson et al. 1990; Zwetyenga, Rogier et al. 1998; Snounou, Zhu et al. 1999). Another major use of *msp* genotyping in antimalarial trials is to distinguish recrudescence from new infection due to treatment failure (Mugittu, Adjuik et al. 2006).

Severity of malaria has been associated with the occurrence of parasite genotypes described by the above described polymorphic genetic markers of the genes for merozoite surface protein 1 and 2 (Tanabe, Mackay et al. 1987). The distribution of these allelic variants has also been shown to vary with geographical location. RO33 was observed in *P. falciparum* infected Brazilians and Senegalese (Kimura, Mattei et al. 1990), while in Gabon RO33 was observed in asymptomatic cases and the allelic marker K1 was observed in severe cases (Kun, Schmidt-Ott et al. 1998). On the other hand, K1 is common in asymptomatic cases in East Africa (Babiker, Ranford-Cartwright et al. 1994). *msp*2's 3D7 and FC27 alleles have been associated with severe malaria, higher parasite density and increased multiplicity of infection in a hyperendemic area of India (Ranjit, Das et al. 2005).

The possibility that specific parasite characteristics contribute to severity of disease was initially investigated in a hypoendemic area in French Guiana (Ariey, Hommel et al. 2001). Parasite genotyping in geographically and temporally matched patients with mild and severe disease showed the association of a specific MSP1 allele (B-K1) with the presence of a particular *var* antigen (a member of a *P. falciparum* multigene family discussed below). *var*-D was over-represented among patients with

severe versus mild disease (47% vs. 3% respectively). These findings suggest an association between parasites possessing certain genes and virulence of disease.

Kun *et al*. 2002 demonstrated that the appearance of a new genotype in a previously steady pattern of asymptomatic infection within a single host coincides with clinical symptoms. However, the same group claims to have shown for the first time that there is no apparent change in *P. falciparum* subpopulations in individual patients during an episode of malaria (Kun, Missinou et al. 2002). They suggest that although an individual in high transmission areas is usually infected by multiple parasite genotypes, the malaria symptoms are caused by one presumably virulent dominant parasite genotype which probably multiplies rapidly and inhibits the growth of concurrent parasites present in the host (Figure 1.6). Some parasite clones more sensitive to the treatment administered to patients during infection are likely to disappear earlier from the circulating blood. The remaining genotypes may be hard to detect if parasitaemia is effectively reduced by the drug (Farnert, Rooth et al. 1999; Franks, Koram et al. 2001; Missinou, Kun et al. 2004).

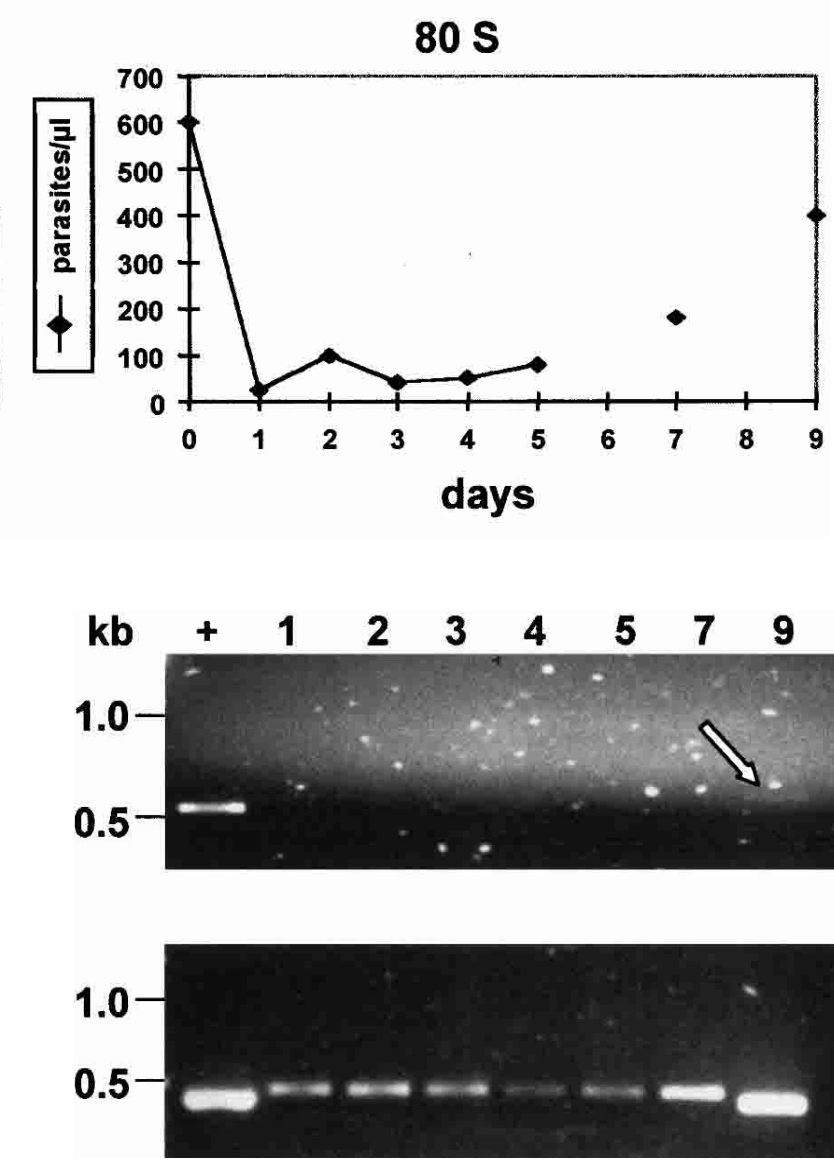


Figure 1.6. Parasite density and genotyping analysis of finger-prick blood sample from an individual child (80S). The **upper** panel shows the course of the parasitaemia (parasite/µL over time (days). The **middle** and **lower** panel shows the results of the PCR products specific for two different *mps*2 alleles; 3D7 family and the FC27 respectively. A molecular size kilobase (kb) ladder was also used. + indicates the positive control for the respective isolate; - indicates the negative control. Lane 9 represents the symptomatic phase. This child experienced 8 days of asymptomatic infection with only one parasite of the FC27 family. On day 9 the child became symptomatic and a unique FC27 variant and an additional 3D7 strain appeared. The **arrow** points to a band representing a minor fraction of 3D7 parasites. (*From Kun et al. Am J Trop Med Hyg 66(6) 2002 653-8*)

Most longitudinal studies on parasite subpopulations in individual patients have been carried out on asymptomatic parasite carriers. Färnert *et al*. 1997 studied the daily dynamics of *P. falciparum* subpopulations in asymptomatic children in Tanzania and reported highly complex changes in parasite density and genotype patterns over time (Farnert, Snounou et al. 1997). This led to the conclusion that the parasites observed in any single blood sample represent only a part of the total body-parasite mass, because the genetic make-up of the parasite population can be very mixed and some parasites are removed from circulation by sequestering in the tissues.

Furthermore, the distribution of some *msp*1 alleles has been shown to be influenced by age (Ntoumi, Rogier et al. 1997). These results are consistent with the interpretation that acquired anti-*P. falciparum* immunity reduces parasite density, limits the number of parasite genotypes infecting an individual at any given time, and controls parasites against which a strong immune response has been mounted (Zwetyenga, Rogier et al. 1998).

1.6.1 Parasite population and genetics in Malawi

Most *P. falciparum* infections are comprised of multiple genetically distinct parasite variants arising from more than one infectious mosquito bite, or a single blood meal transmitting multiple parasite variants (Talisuna, Okello et al. 2007). Infections with a single to a few variants are common in low transmission areas such Asia and Latin America (Juliano, Ariey et al. 2009). In areas of high transmission such as sub-

Saharan Africa, individuals can be detected having more than 10 genetic variants (Greenhouse, Myrick et al. 2006; Kwiek, Alker et al. 2007).

Malawi is one of many sub-Saharan countries with a high burden of malaria, where transmission is perennial and, despite the up-scaling of malaria control measures, there has been no decrease in the incidence of childhood malaria (Roca-Feltrer, Kwizombe et al. 2012). Malawian paediatric *P. falciparum* infections are dominated by clones which persist in both circulation and in parasites sequestered in the tissues (Dembo, Phiri et al. 2006; Montgomery, Milner Jr. et al. 2006) as shown by data generated from other countries using peripheral blood samples (Missinou, Kun et al. 2004). The specific genetic populations defined in terms of *msp*1 and 2 genotypes are not associated with preferential sequestration in the brain in children with malaria. These results have been confirmed by other genotyping methods using a 24 single nucleotide polymorphism assay (Milner, Valim et al. 2012) and massively parallel pyrosequencing (Juliano, Porter et al. 2010). The latter method has revealed up to six-fold more variants per infection, exceeding the results from the currently recommended *msp*1 and 2 genotyping.

Other high resolution methods have also shown that genotypes isolated from Malawian children are conserved within the population with about 15% extreme relatedness within a single patient with heterogeneous infection compared to 21.1% relatedness in parasites from Thailand (Nkhoma, Nair et al. 2012). These results for Malawi are comparable to other highly malaria endemic countries like Tanzania which also show higher mean multiplicity of infection (MOI) for both markers of *msp*1 and *msp*2

genes and increased genetic diversity (Schoepflin, Valsangiacomo et al. 2009). However, the genetic diversity was also not very different from PNG which has low malaria endemicity. Considering the different inoculation rate of >300 infective bites per person per year (Smith, Charlwood et al. 1993; Charlwood, Smith et al. 1998) and average of 5 detected infections per child (Felger, Irion et al. 1999) for Tanzania versus 35 infective bites for PNG (Smith, Hii et al. 2001), this indicates that there was little genetic differentiation between parasite populations in Tanzania and PNG, suggesting that the observed pattern of allele frequencies are independent of transmission intensity (Schoepflin, Valsangiacomo et al. 2009).

The complexity of *P. falciparum* genetic diversity can also be influenced by age (Bendixen, Msangeni et al. 2001). Farnert *et al* showed that while most laboratories might have the same sensitivity and specificity with the *msp*1/2 genotyping method, technical differences can also lead to results that show variation rather than epidemiological differences (Farnert, Arez et al. 2001). Increased intensity of malaria control programmes are also likely to have an effect on parasite genetic diversity. For example, Chikwawa, one of the districts in Malawi with highest malaria transmission shows a significant reduction in moderate anaemia, from 10% to almost 0% due to constant use of IRS for a year (Figure 1.7; Sanie Seseye, personal communication). There was also a reduction of sporozoite infection rate, from 6.4% to almost 0% for *An. fu*ne*stus* and from 6.1% to 0% for *An. gambiae* after IRS intervention, compared to a sporozoite rate of 10.2% in 2005 before IRS intervention (Benjamin Nyoni, personal communication).

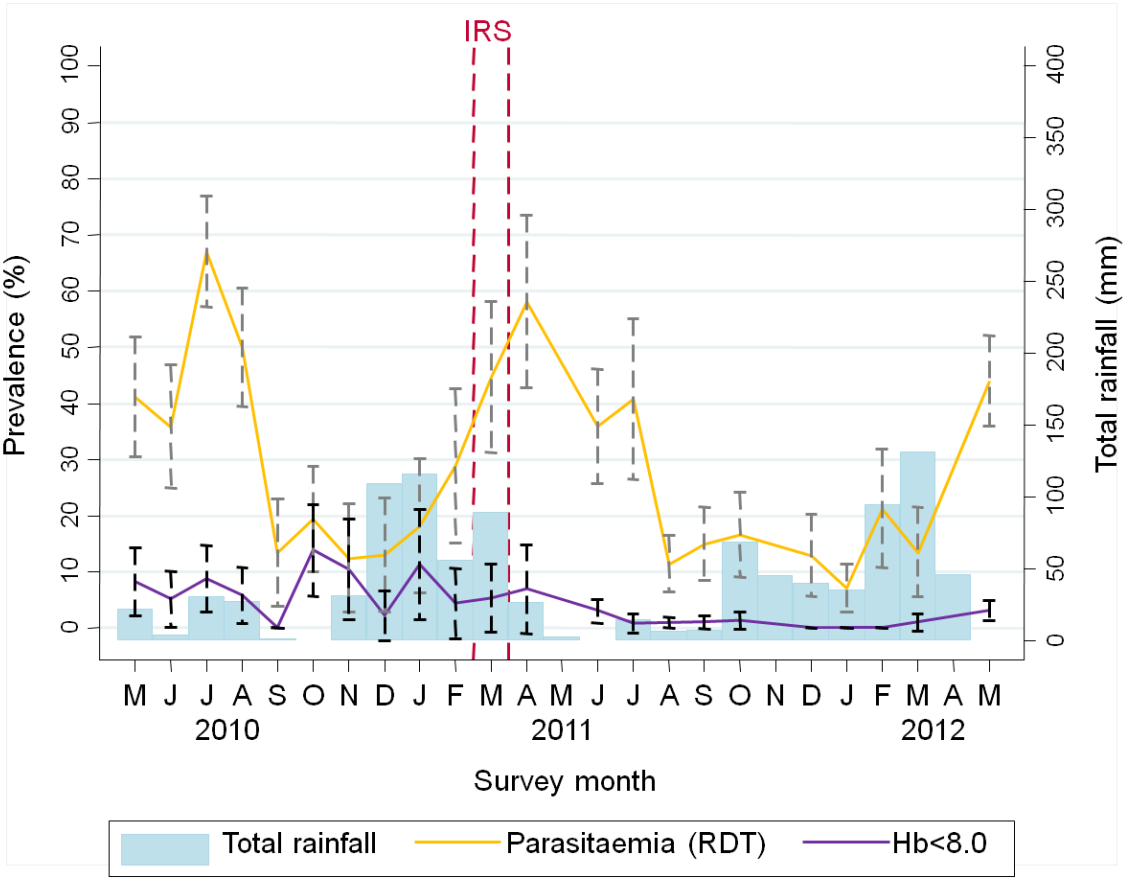


Figure 1.7. Intervention coverage and prevalence of *P. falciparum* parasitaemia and anaemia in children at the study site in Chikwawa district from 2010 to 2012. The study area consists of 50 villages. Since the introduction of IRS in March 2011, parasitaemia in children has dropped to about 10% and malaria–associated anaemia is almost non-existent at 0% prevalence. (*Sanie Sasey, unpublished data*)

1.7 Malaria pathogenesis

1.7.1 Sequestration and cytoadherence

The pathogenicity of *P. falciparum* is thought to result in part from the unique ability of pRBC to adhere to, and activate, vascular endothelium. Mature trophozoites and schizonts are removed from the peripheral circulation (Bignami and Bastianelli 1889), due to adhesion of the infected erythrocytes (Miller 1969) to post-capillary venules, leading to sequestration of pRBC in the tissues. The parasite remodels the pRBC both inside and outside to facilitate its protein trafficking pathways, which result in the deformation of the erythrocyte plasma membrane. Not all of the remodelling proteins are antigens. Some lie underneath the knobs and some remodel the trafficking pathways internally. The antigenic proteins mediate cytoadherence to variety of host endothelial receptors (Luse and Miller 1971).

Cytoadherence can cause occlusion of small vessels, impaired oxygen delivery and hence can contribute to organ failure in the host (Miller, Good et al. 1994). It is also suggested that cytoadhesion aids parasite maturation due to their preference for the microaerophilic venous atmosphere and it allows the parasite to escape clearance by the spleen (Saul 1999). The spleen removes erythrocytes with reduced deformability, such as parasite-infected cells (Looareesuwan, Ho et al. 1987), during acute falciparum malaria as well as selectively extracting parasites from their erythrocytes and leaving the residual cells within the circulation, a mechanism also known as “pitting” (Schnitzer, Sodeman et al. 1972; Anyona, Schrier et al. 2006).

As has been stated above, *P. falciparum* infections are commonly composed of multiple subpopulations of parasites with varied adhesive properties. Variants have been revealed to differ in their efficiency of binding to a range of host receptors and will compete for adhesion to endothelia, suggesting that a mixed infection will not show uniform cytoadherence properties and so parasite variants may vary in their ability to cause pathology (Phiri, Montgomery et al. 2009).

Cytoadherence can activate intracellular signaling pathways in both endothelial cells and pRBC, leading to expression of immune mediators such as cytokines, which can modify the outcome of the infection through monocyte and platelet recruitment, additional cytokine release, and fibrin deposition that aggravates inflammation (Turner, Morrison et al. 1994; Miller, Hudson-Taylor et al. 2002). Furthermore, there has been some evidence of an association between clinical syndromes and adhesion to these receptors (Udomsangpetch, Taylor et al. 1996; Newbold, Warn et al. 1997). Therefore, understanding the molecular events involved in these adhesive interactions is critical in terms of both the pathogenesis and implications for therapeutic intervention.

1.7.2 Variant proteins expressed on the surface of pRBC and their functions

When *P. falciparum* infects the host erythrocyte, it deposits protein on the surface of the pRBC. These proteins are exported from the intracellular parasite through the erythrocyte cytoplasm to the surface via a complex system of vesicle trafficking pathways (Pouvelle, Gormley et al. 1994). There is an array of parasite-

derived proteins identified as being associated with the pRBC cell membrane at various stages of the developmental cycle. *P. falciparum* erythrocyte membrane proteins 1, 2 and 3 (PfEMP1, PfEMP2 and PfEMP3), *P. falciparum* histidine-rich protein (PfHRP1) and knob-associated histidine-rich protein (KAHRP) are associated with knobs. Of these, PfEMP1 is the only protein that extends beyond the cell surface to mediate cytoadherence (Magowan, Wollish et al. 1988), whereas PfEMP2 , PfEMP3 and PfHRP1 or KAHRP remain on the internal face of the erythrocyte membrane in association with electron-dense material.

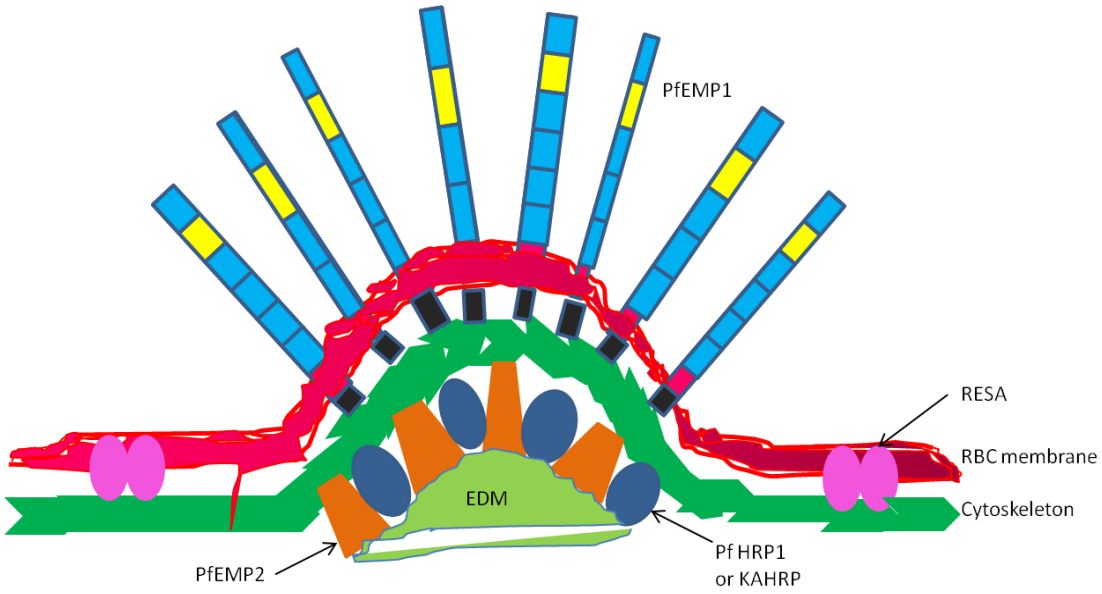


Figure 1.8. Schematic diagram of *P. falciparum* protein distribution on the surface membrane of infected erythrocytes. Lipid bilayer of red blood cell (RBC) membrane is indicated, together with the cytoskeleton and electron-dense material (EDM) under the knob. *P. falciparum* erythrocyte membrane proteins 1 and 2 (PfEMP1 and PfEMP2) and *P. falciparum* histidine-rich protein (PfHRP1) or knob-associated histidine-rich (KAHRP) are associated with knobs. RESA is the ring-infected erythrocyte surface antigen.

The role of these proteins is not fully known; however, PfEMP2 is presumed to have a structural function (Howard, Barnwell et al. 1988). PfEMP3, also found on the surface of sporozoites and in the cytoplasm of mature hepatic stages (Gruner, Brahimi et al. 2001), is thought to be involved in sporozoite invasion. PfEMP3 may also facilitate PfEMP1 function as its mutation can result in the disruption of PfEMP1 transfer to the RBC surface (Waterkeyn, Wickham et al. 2000). On the other hand, because of its ability to produce vesicles, HRP1 is presumed to destabilise the cytoskeleton to allow lateral movements of some membrane components necessary for inward expansion and the formation of the parasitophorous vacuole membrane (Hommel and Semoff 1988).

There are two other parasite proteins associated with the erythrocyte membrane that are not localised specifically to the knobs: ring-infected erythrocyte surface antigen (RESA) and *P. falciparum* histidine-rich protein 2 (PfHRP2). PfHRP2 is secreted into the circulation and is currently being exploited for diagnostic assays (Gamboa, Ho et al. 2010). The RESA antigen is transferred from the merozoite to the erythrocyte membrane during invasion where it is thought to be entirely sub-membranous (Aikawa, Torii et al. 1990). RESA is potential ligand for cytoadherence mediated by CD36 (Cranston, Boylan et al. 1984). More recently, RESA has been shown to reduce ring-stage deformability in static conditions (Mills, Diez-Silva et al. 2007), protect the ring stage from vesiculation damaged induced at high temperatures (Silva, Cooke et al. 2005; Pei, Guo et al. 2007) and thus ensuring survival of the parasite (Diez-Silva, Park et al. 2012).

1.7.3 *Plasmodium falciparum* multigene families

Multigene families (Figure 1.9) encode for *P. falciparum* variant proteins and are considered as potential contributors to antigenic variation which will be discussed in detail in section 1.8. These include the repetitive interspersed family (*rif*); the subtelomeric, variable open reading frames (*stevor*); *P. falciparum* Maurer's Cleft-2 Transmembrane family (*Pfmc-2TM*) and *var* genes (Weber 1988; Cheng, Cloonan et al. 1998).

1.7.3.1 The *rif* multigene family

RIFIN proteins are encoded by the *rif* (repetitive interspersed family) multigene family, ranging between 30-40 kDa in size (Fernandez, Hommel et al. 1999; Kyes, Christodoulou et al. 2003). RIFINS are divided into A and B types. A-type RIFINs are associated with MC while B-type RIFINs are restricted to the parasitic cytosol (Gardner, Hall et al. 2002; Petter, Haeggstrom et al. 2007). Bioinformatic analysis of *rif* genes reveals a sequence conservation of its several members cross HB3, 3D7, IT and Dd2 isolates, with 30 to 50% amino acid identity for any two random RIFINs and >90% amino acid identity between at least one pair of those found in at least three out of the four isolates (Claessens, Ghumra et al. 2011). Like *var* genes (discussed in section 1.7.3.5), *rif* genes undergo switching and clonal expression to produce polymorphic display of RIFINs in the merozoite stages that are fundamental for the parasite to establish a chronic infection (Cabral and Wunderlich 2009; Wang, Magistrado et al. 2009). *In vitro, rif* genes transcription switches faster than *var* genes transcription, at a

rate of 45% per generation (Cabral and Wunderlich 2009) and similar to *var* genes, *rif* gene silencing is dependent on the histone deacetylase, with silent *rif* loci associated to histone 3 lysine 9 trimethylation (Lopez-Rubio, Mancio-Silva et al. 2009; Salcedo-Amaya, van Driel et al. 2009).

Initially, RIFINS were correlated with erythrocyte rosetting using the CD31 receptor (reviewed in (Sherman, Eda et al. 2003), which was later shown to be mediated on the parasite by PfEMP1 (Rowe, Moulds et al. 1997; Chen, Barragan et al. 1998). RIFINs initiate a naturally acquired immune response that has been correlated with a significant rapid parasite clearance from circulation upon treatment in Gabon (Abdel-Latif, Dietz et al. 2003). The presence of high levels of anti-RIFIN antibodies in asymptomatic children from the same population provides some evidence that anti-RIFIN antibodies provide a certain degree of protein against malaria infection (Abdel-Latif, Khattab et al. 2002; Abdel-Latif, Dietz et al. 2003).

1.7.3.2 SURFIN

The SURFINs, encoded by the *surf* multigene family are related to the *P. vivax* subtelomeric transmembrane protein 1 (PvSTP1). Other members of this protein are expressed on the surface of merozoites and other erythrocytic stages (Winter, Kawai et al. 2005) and are suggested to be involved adhesion (Tanabe, Mackay et al. 1987) and erythrocyte invasion (Mphande, Ribacke et al. 2008).

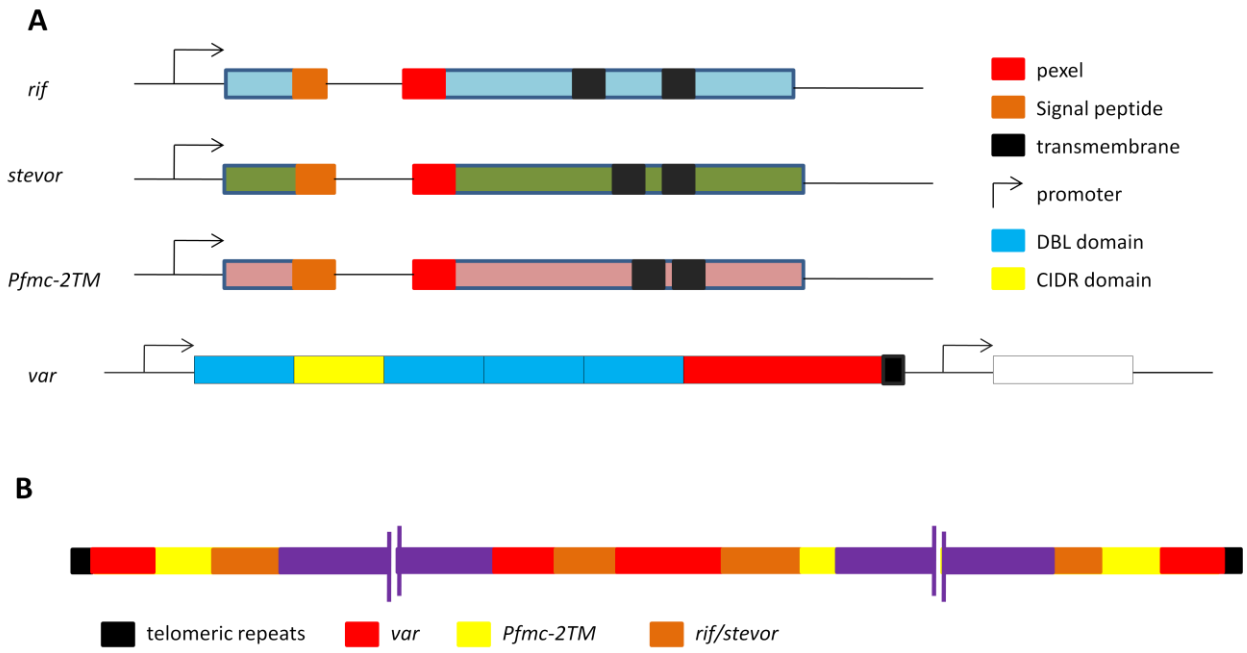


Figure 1.9. Structure and arrangement of variant antigen genes in *P. falciparum.*

A. The gene structure of each of the four major antigen families. The *rifs* , *stevors* and *Pfmc-2TM* all have a two-exon structure with the small first exon encoding the signal peptide, while the second exon contains the PEXEL/VTS motif responsible for erythrocyte trafficking. While *var* genes also have a two-exon structure, they do not contain a signal peptide. In addition, *var* genes have two promoters, the first upstream of exon one and responsible for transcription of the mRNA and the second in the intron, leading to transcription of anti-sense 'sterile' transcripts.

B. Location and arrangement of the *var*, *rif* , *stevor* and *Pfmc-2TM* gene families on a typical *P. falciparum* chromosome. Within the subtelomeric regions, *var* genes are typically located closest to the telomeric repeats, followed by interspersed *rifs* and *stevors*, and lastly by members of the *Pfmc-2TM* family.

1.7.3.3 The *stevor* multigene family

The *stevor* (subtelomeric variable open reading frame) multigene family originally named 7h8 (Limpaiboon, Shirley et al. 1991) consists of 30–40 genes, depending on the parasite isolate, located within *rif*-containing subtelomeric regions on all *P. falciparum* chromosomes. The *stevor* genes are more conserved between parasite isolates than RIFINs. Apart from ultimately being associated with the erythrocyte membrane like RIFINs and PfEMP1, STEVOR are associated with the Maurer's clefts (MC), a unique feature of pRBC that consist of a flat vesicular membranous structure within the erythrocyte cytoplasm that transports proteins across the cytoplasm of the host cell to the surface of the plasma membrane. It is possible that the MC are not the final destination of STEVOR proteins as it has been recently shown that, similar to PfEMP1, they are trafficked through the MC to the surface of the merozoite (Khattab and Meri 2011) and the erythrocyte surface in asexual stages (Kaviratne, Khan et al. 2002; Kissinger, Souza et al. 2002; Lavazec, Sanyal et al. 2006; Blythe, Yam et al. 2008; Khattab, Bonow et al. 2008; Niang, Yan Yam et al. 2009). *Stevors* are also associated with a tubovesicular membrane network that extends from the parasitophorous vacuole through the erythrocyte cytoplasm. These structures have been suggested to be involved in the trafficking of proteins from the parasite to the surface of the pRBC (Przyborski, Wickert et al. 2003).

Although the function of *stevor* remains enigmatic, their localisation within MC suggests their possible functionality within this organelle (Blythe, Surentheran et al. 2004). Their highly variable loop between the two TM domains has already been

implicated in antigenic variation in the face of immune pressure at the erythrocyte surface. Synthetic STEVOR peptides have been shown to bind with high affinity to human erythrocytes (Garcia 2005), suggesting a possible role in forming rosettes and also impacts the deformabilty of pRBC as a tool for sequestration (Sanyal, Egee et al. 2011)

1.7.3.4 The Pfmc-2TM multigene family

The catalogue of 2 transmembrane (2TM) proteins includes several smaller families of paralogous genes (Marti, Baum et al. 2005; Templeton and Deitsch 2005; Sargeant, Marti et al. 2006), most notable of which are 13 genes that encode the Pfmc-2TM proteins (Sam-Yellowe, Florens et al. 2004). The Pfmc-2TM proteins are highly conserved across paralogues within their N-terminal, transmembrane and positively charged C-terminal regions. Indirect immunofluorescence studies have shown that the Pfmc-2TM proteins are localised to the MC (Sam-Yellowe, Florens et al. 2004); however, it is yet to be determined whether the erythrocyte surface is the ultimate, perhaps functional destination of the Pfmc-2TM.

1.7.3.5 The *var* multigene family

By far, the most evident contributors to antigenic variation with biological relevance and also the focus of this study are the *var* genes. One of the success stories in the study of *P. falciparum* is the annotation of the complete genome sequence of the

laboratory *P. falciparum* isolate 3D7 (Gardner, Hall et al. 2002). This achievement and the partial genome sequence of other *P. falciparum* clones HB3 (Volkman, Sabeti et al. 2007) and IT4/25/5 (IT4) permitted the comparison of *var* gene repertoires, with each of the parasite clones representing Africa, Central America, and Southeast Asia, respectively (Kraemer, Kyes et al. 2007). Initially, 61 *var* genes were sequence in 3D7, HB3 had 54 full and partially sequenced *var* genes (including six pseudogenes), and IT4 had 48 full and partially sequenced *var* genes (Kyes, Kraemer et al. 2007) and this data has been matched by recent studies (Rask, Hansen et al. 2010; Claessens, Ghumra et al. 2011).

The *var* genes are scattered on all chromosomes, and the majority of the *var* genes are located in the subtelomeric regions with 22 genes arranged in clusters of tandem repeats in the internal regions in the chromosomes, except chromosome 14 for 3D7 (Gardner, Hall et al. 2002). The three isolates share seven protein architectural types and most *var* genes have overall amino acid sequence identity of <50% within individual domains, even those within the same architectural type. However, three *var* genes (*var1csa*, *var2csa*, and Type 3 *var*) are highly conserved at >75% identity over multiple domains (Kraemer, Kyes et al. 2007).

Overall, all *var* genes have a similar structure, consisting of a long exon 1 that encodes the variable extracellular portion of the PfEMP1 protein (discussed in section 1.7.3.5.2 below), exhibiting both sequence diversity by varying combinations of the Duffy-Binding Ligand (DBL) and Cysteine Rich Inter-domain Region (CIDR) domains. Each pRBC expresses only one PfEMP1 variant by one *var* gene out of the possible 60

var genes present in the parasite genome. However, the parasite is able to switch its PfEMP1 variant though antigenic variation (discussed in section 1.8) by switching *var* gene expression at each new asexual blood stage, potentially giving rise to a phenotype with different adhesive properties. PfEMP1 variants are elevated on the surface of the host erythrocytes through knobs, which are protuberances on infected erythrocyte membrane that appear in early trophozoites stages of the asexual life cycle of the parasite containing various malaria proteins.

All *var* genes also possess an intron that has a highly conserved nucleotide sequence, and a short exon2 that encodes a short intracellular domain that is thought to anchor the protein within the knob structure at the cytoplasmic face of the erythrocyte membrane (Baruch, Pasloske et al. 1995; Su, Heatwole et al. 1995). Each *var* gene possesses two separate promoters, one upstream of exon 1 that is responsible for expression of the messenger ribonucleic acid (mRNA) and is active early in the cell cycle, from 12 to 18 h after invasion and a second promoter within the intron that leads to expression of a non-coding anti-sense RNA mainly by the late-stage parasites and is also thought to regulate gene expression (Calderwood, Gannoun-Zaki et al. 2003; Kyes, Christodoulou et al. 2003). More experimental data is required to elucidate the possible role of the non-coding RNAs in *var* regulation. However, it is speculated that the non-coding RNAs could be involved in chromatin assembly, as has been shown for non-coding RNAs in other systems (Morey and Avner 2004).

1.7.3.5.1 *Var* grouping and classification

Sequence analysis of the upstream regulatory regions of all 3D7 isolate *var* genes have identified three main subgroups of the family: A, B and C and two intermediate groups (A/B and B/C) depending upon sequence motifs in their 5' non-coding regions (ups); and their chromosomal location and orientation of transcription (Kraemer, Gupta et al. 2003; Kraemer and Smith 2003; Lavstsen, Salanti et al. 2003). Rask *et al* has also been able to match all previously described ups subgroups (upsA1–2, upsB1–4, upsC1–2 and upsE) and identified four additional subgroups (upsA3 and upsB5–7) (Rask, Hansen et al. 2010). The three major group classifications based on ups sequence also correspond to HB3, Dd2 and IT4 *var* genes but differences in chromosomal location between isolates argue for a modification of the sub-groupings. For example, the HB3 UpsA1-associated *var* gene (*HB3var6*) is located in a central chromosomal cluster rather than the typical sub-telomeric location.

Group A *var* genes are generally longer and more structurally diverse, with 2-7 DBL domains (Figure 1.10A). However, they also include some of the shortest genes like the Type 3 *var,* which consist of an unusual DBL1α_1–DBL2ε domain combination and share almost no sequence identity with other members of the *var* gene family (Gardner, Hall et al. 2002; Kraemer, Kyes et al. 2007). The proportion of *var* genes in each ups type is similar between 3D7, HB3 and IT4. However, the differences in chromosomal location between isolates call for a modification of the sub-groupings. Due to these difference Kraemer et al devised a naming system based on both *var* gene

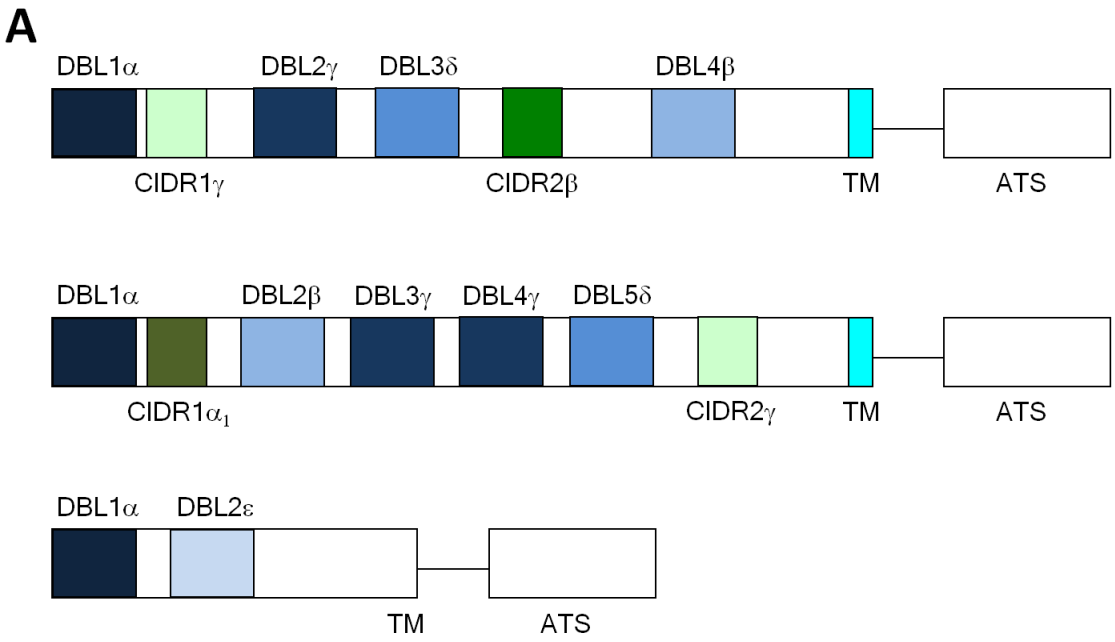
location and ups sequence to allow for the future addition of 'atypical' genes (Kraemer, Kyes et al. 2007).

The largest *var* group is Group B, with 22-27 genes in the *P. falciparum* isolates sequenced so far (Lavstsen, Salanti et al. 2003). They are mostly located in subtelomeric regions, where they are translated towards the centromere, or in central chromosomal regions. Most genes in this group consist of a typical DBL1α-CIDR1α head structure followed by a single DBL2δ and a non-α CIDR2 domain (Figure 1.10B). Group B genes can be further divided into 4 sub-types (B1-4) based on upsB sequence variations (Kraemer, Kyes et al. 2007).

Group C *var* genes also exhibit the common DBL1α–CIDR1α–DBL2δ–CIDR2non-α gene structure and they are exclusively located in centromeric regions (Figure 1.10 C) (Kraemer, Kyes et al. 2007). Their upstream sequences (upsC) are diverged from the other gene groups and found in 2 variations (C1-2). Interestingly, genes containing the upsC upstream sequences have been shown to also be expressed during gametocyte development (Sharp, Lavstsen et al. 2006).

Initially, two other *var* gene groups, D and E, were identified. These were represented by one gene each in 3D7 and paralogues shared over 75% identity in multiple domains and over 90% in some stretches (Gardner, Hall et al. 2002; Kraemer, Kyes et al. 2007). Group D was represented by the longest gene identified thus far as the *var1csa* pseudogene. Its upstream sequence has since been shown to cluster phylogenetically with upsA sequences and thus this group has been reclassified as the A2 group, whereas all other upsA genes are A1 type (Kyes, Christodoulou et al. 2003).

Group E is the *var2csa* gene, found at 1 copy in the 3D7 and IT4 isolates and in 2 copies in HB3. It is unusually structured with 3 DBLx domains followed by 3 DBLε domains (Figure 1.10) (Gardner, Hall et al. 2002). *Var2csa* have very little to no sequence identity with other members of the *var* gene family. This gene is expressed at high levels in placental-associated malaria and is thought to be the main ligand used in interacting with CSA, expressed on syncytiotrophoblasts in the placenta and is a major vaccine candidate for this form of malaria (Salanti, Dahlback et al. 2004).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DBL2γ
DBL3δ
DBL4β
CIDR1γ
CIDR2β
TM
ATS
DBL1α
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TM
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DBL2ε
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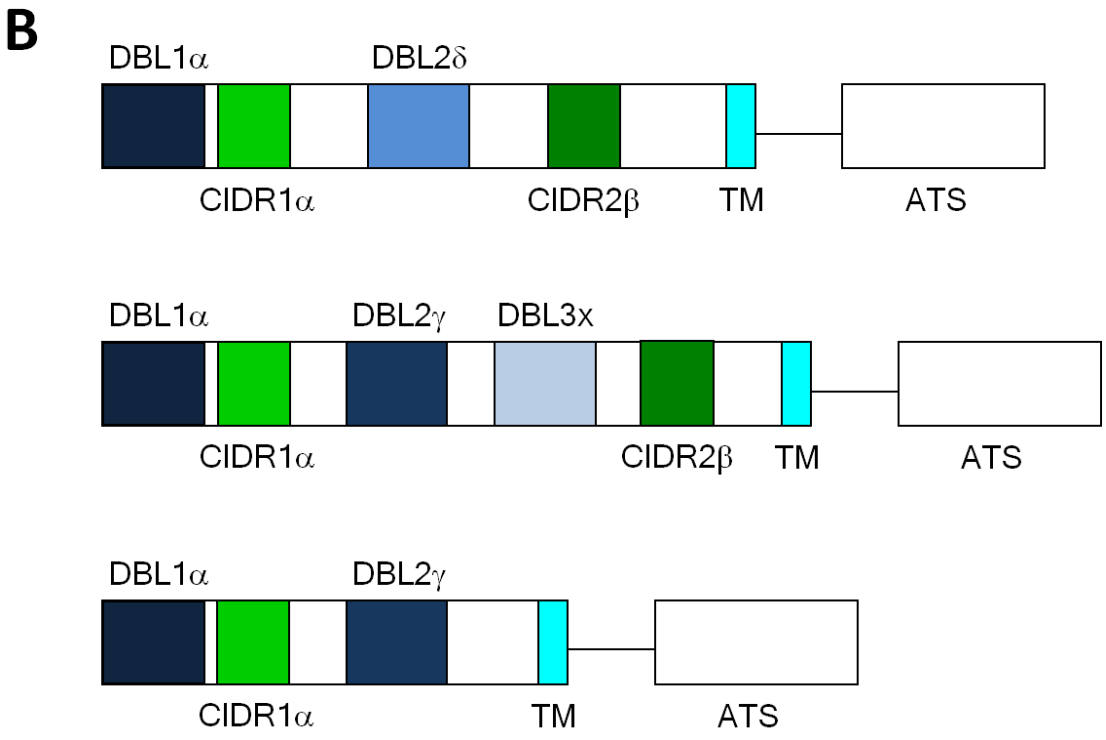
B
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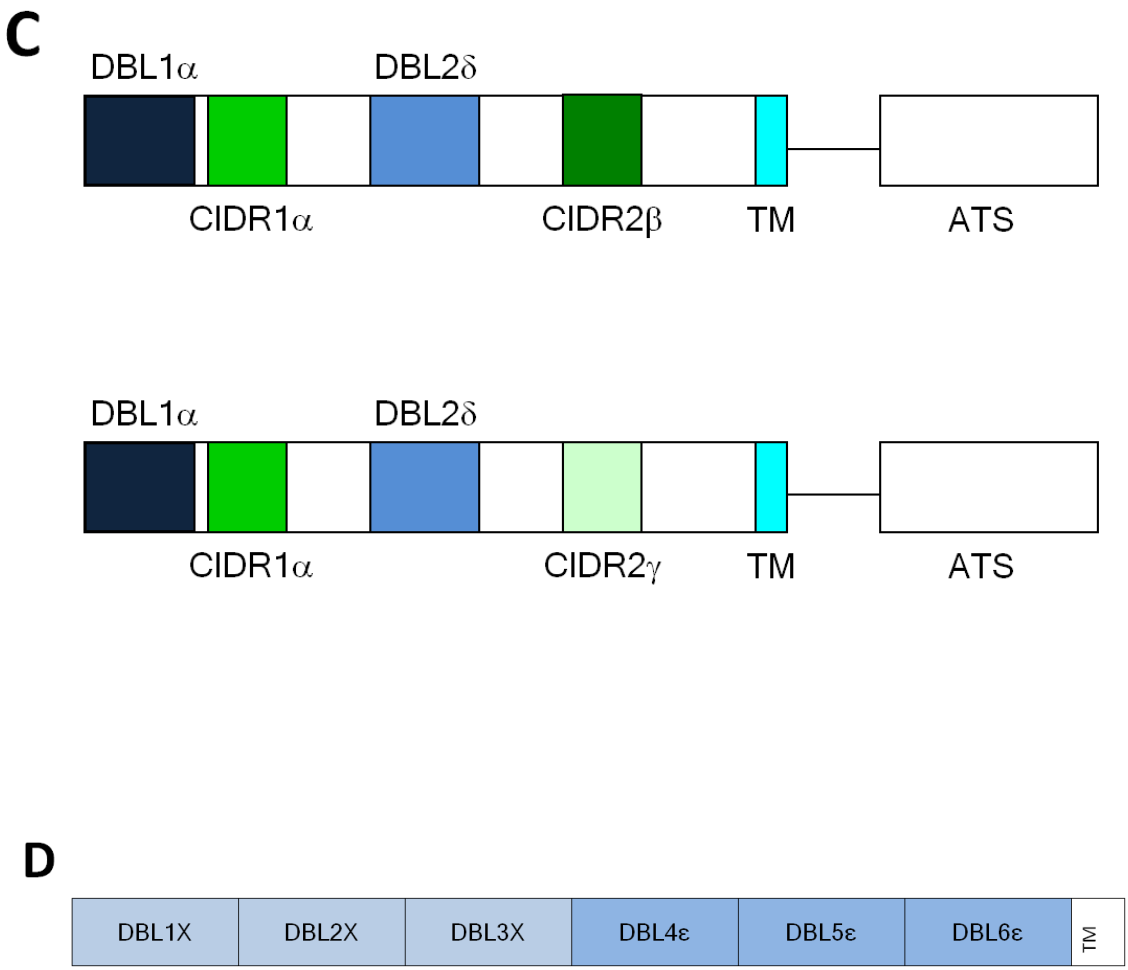


Figure 1.10. Var groups and their domains. A. Group A *var* genes are generally longer and more structurally diverse, with 2-7 DBL domains. They do consist of some of the shortest genes like the Type 3 *var,* which consist of an unusual DBL1α_1–DBL2ε domain combination. **B.** Group B is mostly located in subtelomeric regions where they are translated towards the centromere. Most genes in this group consist of a typical DBL1α-CIDR1α head structure followed by a single DBL2δ and a non-α CIDR2 domain. **C.** Group C *var* genes also exhibit the common DBL1α–CIDR1α–DBL2δ–CIDR2 non-α gene structure and they are exclusively located in centromeric regions. **E.** *Var2csa*

1.7.3.5.2 P. falciparum erythrocyte membrane protein 1 (PfEMP1)

PfEMP1 are highly polymorphic antigens displayed within electron dense knobs on the surface of pRBC (Leech, Barnwell et al. 1984; Magowan, Wollish et al. 1988; Baruch, Pasloske et al. 1995). They are large proteins ranging between 200-350 kDa and are encoded by a multicopy gene family collectively named *var*. Each parasite genome contains about 60 *var* genes (Baruch, Pasloske et al. 1995; Smith, Chitnis et al. 1995; Su, Heatwole et al. 1995; Gardner, Hall et al. 2002). PfEMP1 molecules (Figure 1.11) have a general structure that is composed of a long extracellular region containing several conserved structural features: an N-terminal segment (NTS); Duffy-Binding-Like-Domains (DBL; α-ζ); cysteine rich inter-domain regions (CIDR; α-γ); a transmembrane domain (TM) and a conserved intracellular acidic terminal segment (ATS) (Su, Heatwole et al. 1995; Smith, Subramanian et al. 2000). According to the classifications by Rask *et al* the DBL domain has five smaller distinct classes; four of which are based on the N-terminal DBL domains of *var2csa* (Lavstsen, Salanti et al. 2003), and the DBLα of *var*3. The CIDR domain has two additional classes; δ, and pam which include the inter-domain 2 of *var2csa*. NTS sequences are divided into three classes, NTSA, NTSB, and NTSpam while ATS sequences are divided into ATSA, ATSB, ATSpam, ATS*var*1, and ATS*var*3 (Rask, Hansen et al. 2010).

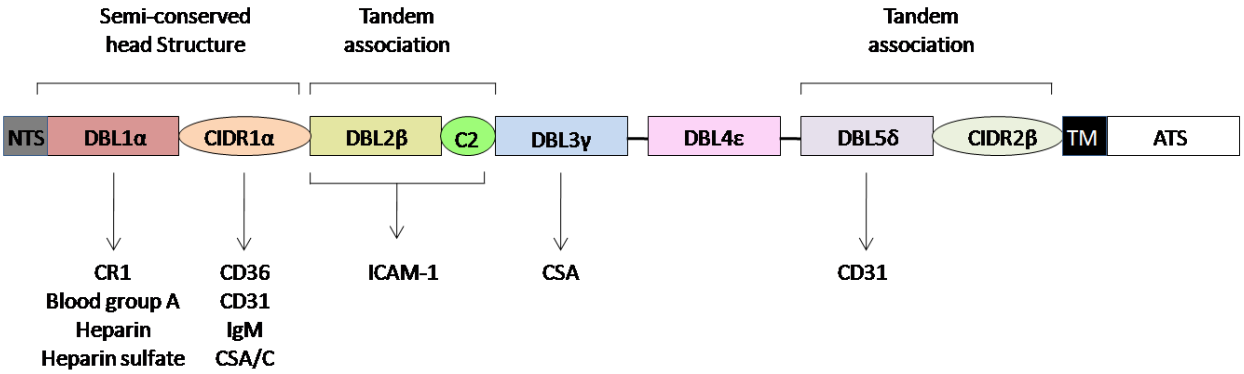


Figure 1.11. Schematic structure of PfEMP1 showing the major domains some of the proposed host receptors they bind. Duffy-Binding-Like-Domains (DBL; α-ε), cysteine rich inter-domain regions (CIDR; α,β), N-terminal segment (NTS), constant region (C2), Transmembrane (TM) and acidic terminal segment (ATS).(*Adapted from Smith et al, Trends in Parasitol 17(11) 2001 538-545*)

All the CIDR1α from the general PfEMP1 sequences bind to the CD36 receptor (Baruch, Ma et al. 1997; Gamain, Smith et al. 2001; Robinson, Welch et al. 2003). The most conserved domains of the PfEMP1 structure are the DBL1 and the C-terminal ATS region. The overall similarity in amino acid sequence of the DBL1 domain is >53%. However, sequences of domains of DBL1α are more common and more diverse while those of rarer DBL1α_1 domains are more closely related which could be an indication of functional differences (Robinson, Welch et al. 2003). Although all *var* genes maintain the basic architecture, the amino acid sequence is highly variable when comparing PfEMP1 proteins among paralogues and across parasites isolates, indicating that there exists a virtually unlimited repertoire of PfEMP1 variants depending upon transmission intensity and geographical location (Kyes, Taylor et al. 1997; Fowler, Peters et al. 2002;

Trimnell, Kraemer et al. 2006). This high level of sequence diversity is thought to be maintained through gene conversion and recombination events within the family (Deitsch, del Pinal et al. 1999; Freitas-Junior, Bottius et al. 2000; Flick and Chen 2004).

PfEMP1 has been identified as one of the major ligands to mediate the unique ability of the mature blood stage parasites of *P. falciparum* to adhere to capillary and post-capillary venular endothelia during the second half of the 48 hour life cycle (MacPherson, Warrell et al. 1985). Their adhesive properties are considered to be one of the major contributors to virulence, as isolates with minimal capacity to bind to host receptors cause mild or unapparent infections in animal models (Langreth and Peterson 1985). The role of the spleen to control parasite burden and protect against severe manifestations of malaria is well-appreciated in splenectomised malaria patients who develop actute *P. falciparum* malaria with high parasitaemia (Pongponratn, Viriyavejakul et al. 2000; Demar, Legrand et al. 2004; Bach, Baier et al. 2005). In addition, the blood film miscoscopy reveals the presence of mature asexual stages which that do not sequester would otherwise be removed from circulation by a functional spleen if present (Bach, Baier et al. 2005; Bachmann, Esser et al. 2009) and by the spleen modulating sequestration by unknown mechanisms (David, Hommel et al. 1983; Pongponratn, Viriyavejakul et al. 2000; Buffet, Safeukui et al. 2011). Some PfEMP1 adhesion interactions are proposed to lead to severe disease manifestations. A good example of this is the *var2csa* that has been identified as the main receptor mediating parasite sequestration in placental malaria (Silamut, Phu et al. 1999; Chen, Schlichtherle et al. 2000).

The position of the PfEMP1 on the pRBC enables the antigen to interact with various receptors, thus mediating adhesion with their multiple binding domains. However, this also makes it particularly vulnerable to recognition by antibodies produced by the host. Each *var* gene encodes an antigenically distinct form of PfEMP1, and supports adhesion to a range of possible endothelial receptors (Gardner, Pinches et al. 1996; Chen, Heddini et al. 2000). The extremely high level of PfEMP1 sequence diversity is a highly effective strategy to avoid host immune evasion and parasite clearance by the spleen, thus ensuring the survival of blood stage infections (Looareesuwan, Ho et al. 1987; Anyona, Schrier et al. 2006).

1.8 Antigenic variation

Bacterial, protozoan and fungal pathogens have evolved similar mechanisms of antigenic variation to avoid eradication by their host immune system and to maintain persistent infections (reviewed in (Deitsch, Lukehart et al. 2009). In *Plasmodium spp.*, the first indications of antigenic variation were mentioned by Cox (Cox 1959) after observation of relapse in parasite populations in *P. berghei*-infected mice. Years later, antigenically variant *P. knowlesi* was described (Brown and Brown 1965), with the protein responsible for antigenic variation on the surface of infected erythrocytes identified in 1983 (Howard et al. 1983). Primarily, antigenic variation was demonstrated in other *Plasmodium* species that possess a range of antigenic families with different properties: in *P. falciparum* (Langreth and Reese 1979), *P. fragile* (Handunnetti, Mendis et al. 1987), *P. chabaudi* (McLean, Pearson et al. 1982), and in *P.*

vivax (Mendis, Ihalamulla et al. 1988; Cole-Tobian and King 2003; Ntumngia, McHenry et al. 2009).

Of all the malaria species that cause disease in humans, only *P. falciparum* can be maintained in *in vitro* culture long enough to allow extensive study of its variant surface proteins. *Var* genes have been shown to be expressed in a mutually exclusive manner at both the mRNA (Scherf, Hernandez-Rivas et al. 1998; Voss, Healer et al. 2006) and protein levels (Dzikowski, Frank et al. 2006). The paradigm of mutually exclusive expression is a key element that underlies the process of antigenic variation and the ability of parasites to evade the host immune response, ensure blood-stage parasite survival and thereby promote transmission to additional hosts. Only one *var* gene is dominantly expressed in the mature stages of the parasite. A small subpopulation of parasites switch expression of its PfEMP1 variant (Smith, Chitnis et al. 1995) and they find an advantageous niche (while the immune system is distracted by the major variant) and expand, thus resulting in the persistent nature of the disease as well as the waves of parasitaemia frequently involved in *P. falciparum* infections (Miller, Good et al. 1994).

Mature erythrocytes provide an ideal atmosphere for the parasite to hide from the immune system of the host because of their simple morphology. They do not contain internal mechanisms of synthesis or traffic of proteins. They also do not express class I or II MHC molecules on their surface.

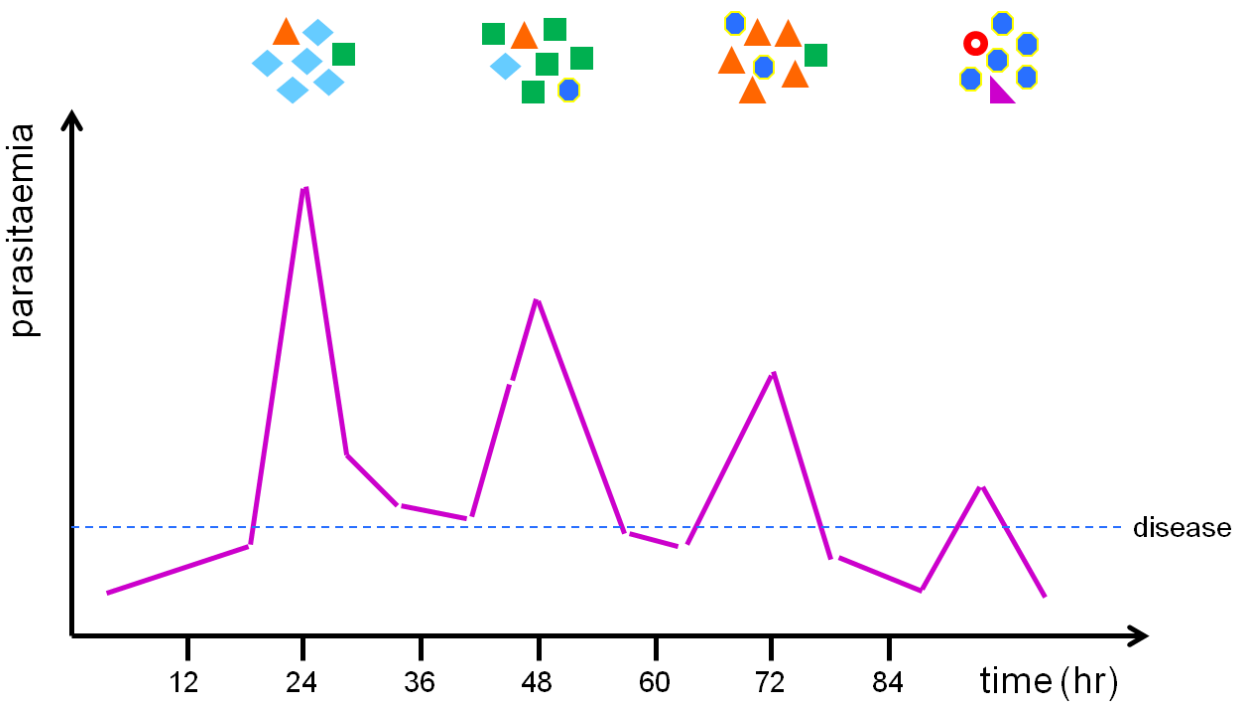


Figure 1.12. Schematic model of antigenic variation of PfEMP1. Each PfEMP1-expressing pRBC is represented by a different colour. Each wave of parasite load is dominated by a single type of PfEMP1 (light blue) at 24 hours. The population switches to a different major PfEMP1 (green) at 48 hours due to host immune response (and possibly drugs). Antigenic variation continues during the course of the disease until all parasites are eliminated.

The host immune system initially develops antibodies against the predominant form of PfEMP1 antigen and then consequently selects for subpopulations that arise from switching of expression between *var* genes. Seemingly, from the 60 *var* genes rises variant PfEMP1s that result in long term persistence of a single infection that varies in magnitude from undetectable levels to high parasite burdens (Miller, Good et al. 1994). After 18 hours of invasion by *P. falciparum*, these surface antigens mediate adhesion to receptors on the host endothelium, preventing the infected erythrocytes

from passing through the spleen. In doing so, the erythrocyte surface proteins make the parasite "visible" to the host immune system at the same time as they withdraw from circulation (Newbold, Craig et al. 1999).

1.9 Host receptors implicated in cytoadherence

The process of cytoadherence involves specific interactions between parasite ligands expressed on the surface of the pRBC and receptors on the host vascular endothelium. A number of endothelial receptors have been identified based on their ability to support the adhesion of laboratory-selected parasite isolates in *in vitro* adhesion assays. Individual receptors are discussed below:

1.9.1 Thrombospondin

One of the first receptors to be identified was thrombospondin (TSP), a protein known to adhere to pRBC in a dose-dependent manner (Roberts, Sherwood et al. 1985; Sherwood, Roberts et al. 1987). There are three potential ligands for TSP: PfEMP1 (Baruch, Gormely et al. 1996), red-cell-derived phosphatidylserine (Eda and Sherman 2002) and altered band 3 protein (Eda, Lawler et al. 1999). Although TSP contributes to cytoadherence, it was later found insufficient to support the process by itself due to low affinity of binding under flow conditions (Panton, Leech et al. 1987).

1.9.2 CD36

CD36 or platelet glycoprotein IV, commonly found on endothelial cells and blood monocytes, is described as one of the main host cytoadherence receptors for pRBC (Barnwell, Asch et al. 1989; Ockenhouse, Ho et al. 1991). CD36 is capable of binding almost all parasites from infected patients (Hasler, Handunnetti et al. 1990; Newbold, Warn et al. 1997) by using CIDRα domains of PfEMP1, encoded by group B and C *var* genes (Robinson, Welch et al. 2003). The ability to bind to TSP and CD36 is highly correlated; however, pRBC adherence to CD36 is a firmer attachment (Asch, Liu et al. 1993). The role of CD36 in malaria pathogenesis is unclear (Serghides, Smith et al. 2003). In African subjects, there has mostly been no difference in CD36 binding ability between parasites isolates from SM and UM patients (Marsh, Marsh et al. 1988; Newbold, Warn et al. 1997; Rogerson, Tembenu et al. 1999; Heddini, Chen et al. 2001). The bulk of current evidence leans towards supporting the role of CD36 in UM (Ochola, Siddondo et al. 2011). A positive correlation was shown between CD36 binding and SM in Thailand (Ho, Singh et al. 1991; Ockenhouse, Ho et al. 1991) but this was not confirmed in a more recent study (Omi, Ohashi et al. 2003). Based on human genetic studies, there is some evidence that CD36 might have a role in SM development but more studies are required (Cserti-Gazdewich, Dhabangi et al. 2012).

1.9.3 ICAM-1

Intercellular adhesion molecule-1 (ICAM-1), an immunoglobulin superfamily adhesion molecule, is another host cytoadherence receptor that adheres to 80% of

isolates. ICAM-1 is distributed widely on vascular endothelium from a range of organs and is the proposed receptor for PfEMP1 in cerebral microvessels because they do not express CD36 (Berendt, Simmons et al. 1989). In support of a role for ICAM-1 in cerebral cytoadherence, pRBC ICAM-1 binding ability has recently been associated with cerebral malaria in Kenya (Ochola, Siddondo et al. 2011). Such associations have never been observed in Asian field isolates (Ockenhouse, Ho et al. 1991; Udomsangpetch, Taylor et al. 1996). In one immunopathological study, co-localisation of sequestered pRBC with ICAM-1 expression was noted, particularly in the brain (Turner, Morrison et al. 1994). However, post-mortem studies need to be interpreted with caution, as adhesion molecules could be expressed as a consequence of the circulatory disturbance and metabolic abnormalities resulting from death rather than being its cause. For example, oxidant damage by adherent sickle cells has been shown to induce the expression of ICAM-1, E-selectin, and VCAM-1 on vascular endothelium (Sultana, Shen et al. 1998).

ICAM-1 is widely upregulated by cytokines: tumor necrosis factor (TNF), interleukin-1 (IL-1) and interferon-γ (IFN-γ) (Pober 1987). It binds the DBL2βC2 domain of PfEMP1 (Smith, Craig et al. 2000; Chattopadhyay, Taneja et al. 2004; Springer, Smith et al. 2004) mostly encoded by the group B *var* genes (Howell, Levin et al. 2008).

1.9.4 CSA

Chondrotin sulphate A (CSA), a carbohydrate expressed on syncytiotrophoblasts that line the placental intervillous space and found in association with

thrombomodulin, has been identified as the main receptor for placental sequestration in PM (Fried and Duffy 1996). CSA is also widely expressed at different levels throughout the human body but displays weak adherence to pRBC. The blood flow in the placental intervillious space is very slow, an advantage for the parasite to firmly adhere to *var2csa* as the major ligand (Salanti, Staalsoe et al. 2003).

Hyaluronic acid (HA) has also been suggested as an additional receptor for sequestration of pRBCs in the placenta. However, it has shown low affinity binding under flow conditions which suggest that it might not be the main cause of pRBC accumulation in the placenta (Beeson, Rogerson et al. 2000). The involvement of HA in placental sequestration is controversial, with claims that the original HA extracts showing adhesion were contaminated with CSA while others claim that the contamination was very minute to cause a significant effect. Recently, both CSA and HA have shown to bind *P. vivax* under static and flow conditions (Chotivanich, Udomsangpetch et al. 2012).

1.9.5 Immunoglobulin-like receptors

Other members of the immunoglobulin superfamily are implicated in pRBC binding: vascular adhesion molecule-1 (VCAM-1; also known as CD106), platelet endothelial cell adhesion molecule 1 (PECAM-1; also known as CD31), E-selectin (CD62E) and P-selectin (CD62P) (Ockenhouse, Tegoshi et al. 1992; Newbold, Warn et al. 1997; Treutiger, Heddini et al. 1997; Chen, Heddini et al. 2000). VCAM-1 and E-selectin

are not constitutively expressed on endothelial cells but rather are induced by IL-1 and TNF. The parasite ligands responsible for adhering to them remain unclear. In African isolates, both VCAM-1 and E-selectin showed low binding and are not associated with disease severity (Newbold, Warn et al. 1997).

P-selectin is expressed on activated platelets and endothelia and is important for leukocyte trafficking. It has been shown to facilitate CD36 binding of Thai field isolates (Udomsangpetch, Reinhardt et al. 1997; Ho, Schollaardt et al. 1998; Yipp, Hickey et al. 2007). PECAM-1 is widely found on endothelia, platelets, monocytes and other immune system cells. The binding of certain field isolates from Kenya to PECAM-1 does not show a significant correlation with SM (Newbold, Warn et al. 1997), whereas a PECAM-1 polymorphism in Thailand isolates showed an increased risked of CM (Kikuchi, Looareesuwan et al. 2001). An analysis of PECAM-1 polymorphisms revealed that the frequencies of certain variants are a risk factor for cerebral malaria (Kikuchi, Looareesuwan et al. 2001). The parasite ligand for both P-selectin and PECAM-1 is unknown but is thought to be PfEMP1 because purified PfEMP1 can bind to P-selectin *in vitro* (Treutiger, Heddini et al. 1997; Senczuk, Reeder et al. 2001) and both CIDRα and DBL2δ domains of PfEMP1 mediate PECAM-1 binding (Chen, Heddini et al. 2000; Yipp, Hickey et al. 2007).

1.9.6 Other potential receptors for cytoadhesion

Although their involvement in cytoadherence is debatable, other potential receptors for *P. falciparum* are collagen (Tandon, Kralisz et al. 1989), high molecular

weight glycoprotein fibronectin (Eda and Sherman 2004), some high and low density lipoproteins. Heparan sulphate has been shown to mediate rosetting and possible binding to CD31 (Calvo, Gomez-Coronado et al. 1998; Chen, Heddini et al. 2000). PfEMP1 also binds to complement receptor (CR1/CD35), found on the surface of non-infected erythrocytes, and ABO blood group antigens to form rosettes which are thought to impair circulation and potentially cause ischemic complications in malaria (Kaul, Roth et al. 1991; Dondorp, Pongponratn et al. 2004). *P. falciparum* rosetting parasites bind IgM natural antibodies ("non-immune" IgM) from normal human plasma/serum (Scholander, Treutiger et al. 1996; Rowe, Shafi et al. 2002). Other endothelial receptors for *P. falciparum* but whose ligands are not yet known include cytokine protein fractalkine (Hatabu, Kawazu et al. 2003), alpha(v)beta3 receptor (Siano, Grady et al. 1998), neural cell adhesion molecule (NCAM or CD56) (Pouvelle, Matarazzo et al. 2007) and gC1qR-HABP1-p32 (Biswas, Hafiz et al. 2007).

1.10 Molecular mechanisms of cytoadhesion

It has been established that the adhesion of pRBC to microvascular endothelium is a central event in the pathogenesis of falciparum malaria. The molecular mechanisms and rheological characteristics of this interaction are of profound importance. Early studies of cytoadhesion were carried out using static adhesion assays, not taking into consideration that *in vivo* pRBC adhesion to vascular endothelial cells is likely to be influenced by the shear forces induced by flowing blood and the kinetics of receptor-ligand coupling. The now widely used flow-based conditions closely mimic the

microcirculation *in vivo* (Nash, Cooke et al. 1992; Cooke, Berendt et al. 1994; Cooke and Coppel 1995).

Studies that have used these techniques have demonstrated the strong dependence of adhesion on flow stress and have also shown clinical parasite isolates interacting with endothelial receptors in a stepwise process; tethering, rolling (slow, end-over-end movement in direct contact with the endothelial surface) and firm adhesion (no detectable movement) (Figure 1.13). Significantly firmer adhesion is observed to CD36 than to ICAM-1 (Nash, Cooke et al. 1992; Cooke, Berendt et al. 1994; Cooke and Coppel 1995). This stepwise process tends to differ depending on the type of receptor. However, the strength of the rolling and interaction with each receptor molecule varies. Some pRBC bypass the rolling event and rest on CD36 immediately after tethering (Udomsangpetch, Reinhardt et al. 1997).

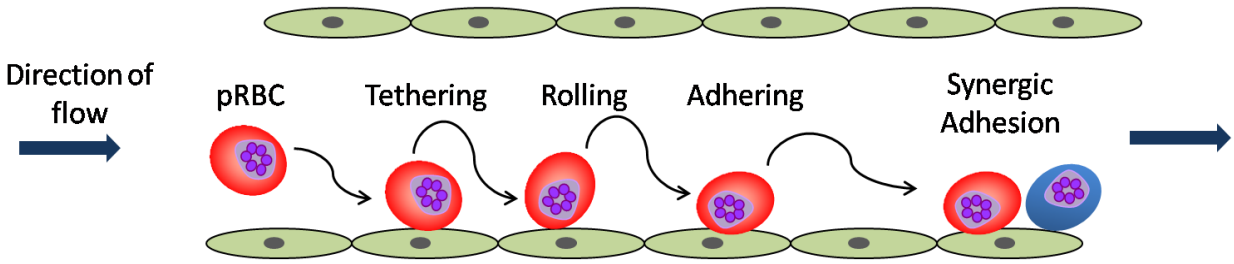


Figure 1.13. Schematic diagram of the different phases of infected erythrocyte interaction with endothelium under physiological flow conditions. The tethering of one parasite (red) provide local disturbance in flow rates allowing the infected erythrocyte with reduced binding ability (blue)to adhere to the endothelium efficiently

P. falciparum variants have been shown to compete for adhesion to endothelia based on their efficiency of binding and it has been assumed that 'efficient' binders will cause severe disease. There are several studies showing correlation between the level of adhesion and the severity of the disease, with severe malaria isolates showing a stronger adhesion to endothelial receptors (Newbold, Warn et al. 1997; Heddini, Pettersson et al. 2001; Phiri, Montgomery et al. 2009) (except for Stephen Rogerson who shows the opposite (Rogerson, Tembenu et al. 1999)). This suggests that variants from a genetically mixed infection will not display uniform cytoadherence and so may vary in their level of pathology in different organs depending on receptor expression.

To determine the relative importance of cytoadherence, pRBC taken directly from the peripheral blood of the patients with acute falciparum malaria have been examined in a number of studies using static and flow binding assays (Hasler, Handunnetti et al. 1990; Ho, Singh et al. 1991; Ockenhouse, Ho et al. 1991; Chaiyaroj, Angkasekwinai et al. 1996; Newbold, Craig et al. 1997; Udomsangpetch, Reinhardt et al. 1997). The degree of adhesion to CD36 is at least 10 fold higher than adhesion to ICAM-1. Minimal or no adhesion to E-selectin, VCAM-1, or CSA is seen with most paediatric isolates. When the degree of cytoadherence to CD36 is compared at a fixed parasitemia, a range of intrinsic cytoadherent capabilities among different isolates becomes evident and in some instances correlates positively with the clinical severity of the infection (Ho, Singh et al. 1991; Ockenhouse, Ho et al. 1991). The high percentage of null cells observed in *in vitro* studies are unlikely *in vivo*, in that mature

parasites are seldom seen in the peripheral blood, yet multiplication is remarkably efficient even in the face of splenic clearance.

P-selectin has been shown to be important for mediating the capture and fast rolling of pRBC followed by slower rolling and attachment to CD36 (Moore, Patel et al. 1995). A likely scenario is that pRBC may need to interact with a number of endothelial receptor molecules for optimal adhesion. The rolling interactions with molecules such as ICAM-1 and P-selectin appear to facilitate adhesion to CD36, even if individually they are of much lower avidity than that required to allow attachment. This is the first demonstration of synergism among receptor molecules for cytoadherence under flow conditions. There is also synergism between CD36 and ICAM-1 in mediating cytoadherence to human dermal microvascular endothelial cells under static conditions and flow-based assays (McCormick, Craig et al. 1997; Gray, McCormick et al. 2003; Phiri, Montgomery et al. 2009).

To date, parasite-host interactions show a complex pattern, with the parasite having a plethora of different host receptors to cytoadhere to. However, field isolates have shown restricted binding, with pronounced differences in host receptor specificity and extent of cytoadherence. These differences provide a scenario where different receptors can act synergistically. Adherence to one receptor can increase binding efficiency to other receptors (McCormick, Craig et al. 1997; Yipp, Anand et al. 2000; Gray, McCormick et al. 2003; Phiri, Montgomery et al. 2009). Synergism may be particularly important in view of the fact that CD36 expression on microvascular

endothelium does not appear to be upregulatable (Petzelbauer, Bender et al. 1993). Therefore, the degree of cytoadherence of *P. falciparum* on vascular endothelium may be regulated at the level of expression of adhesion molecules such as P-selectin and ICAM-1 rather than that of CD36.

1.10.1 Rosetting

Rosetting, the binding of pRBC to uninfected RBC, has been associated with high parasite density in numerous geographic areas and with severe malaria in Africa (Kun, Schmidt-Ott et al. 1998; Rowe, Kyes et al. 2002; Normark, Nilsson et al. 2007). Rosetting is viewed to contribute to pathogenesis due to the occlusion of the microvessels by the resulting agglutinates. The main host parasite ligands suggested for this phenomenon are *var* group A PfEMP1 (Bull, Berriman et al. 2005; Kaestli, Cockburn et al. 2006; Claessens, Ghumra et al. 2011). *P. falciparum* rosetting parasites are placed into two distinct phenotypes: those that bind IgM natural antibodies from normal human plasma/serum onto the surface of IEs (Scholander, Treutiger et al. 1996; Rowe, Shafi et al. 2002) and those that do not. IgM-binding rosetting isolates are common in parasites isolated from children, in whom they are associated with severe disease (Rowe, Shafi et al. 2002). IgM-binding of rosetting parasites is also thought to strengthen the infected-uninfected erythrocyte interaction in rosettes (Scholander, Treutiger et al. 1996; Clough, Atilola et al. 1998; Somner, Black et al. 2000), and also possibly aid parasites in evading immunity by masking key epitopes (Barfod, Dalgaard et al. 2011).

Previous studies have also shown RIFINs to rosette via CR1, heparan sulphate and CD31 (Calvo, Gomez-Coronado et al. 1998; Chen, Heddini et al. 2000). In contrast, Claessens *et al* observed a few *rif* genes highly expressed in a rosetting *P. falciparum* isolate, IT/R29 but their expression was not correlated to the high rosetting frequency (Claessens, Ghumra et al. 2011).

1.10.2 Clumping

The ability of *P. falciparum* clinical isolates to form platelet-mediated clumps has been associated with severe malaria in most studies, and accumulation of platelets in cerebral microvasculature was shown in CM patients but not in those who died of severe malarial anaemia (Pain, Ferguson et al. 2001; Grau, Mackenzie et al. 2003; Chotivanich, Sritabal et al. 2004; Arman, Raza et al. 2007). PfEMP1 has been suggested as the parasite ligand involved in clumping, the aggregation of pRBC and platelets (Pain, Ferguson et al. 2001). Although the molecular binding mechanisms between pRBC and platelets is not yet fully understood, CD36 was originally shown to be the host receptor mediating this interaction, and the ability of CIDRα domains to bind this receptor is well defined (Baruch, Ma et al. 1997; Smith, Kyes et al. 1998; Robinson, Welch et al. 2003). A recent hypothesis suggests that CD36-expressing platelets act as a bridge between pRBC and brain endothelia. The CIDR1 of group B and C PfEMP1 variants are likely to bind to CD36 in contrast to CIDR domains of group A variants (Robinson, Welch et al. 2003).

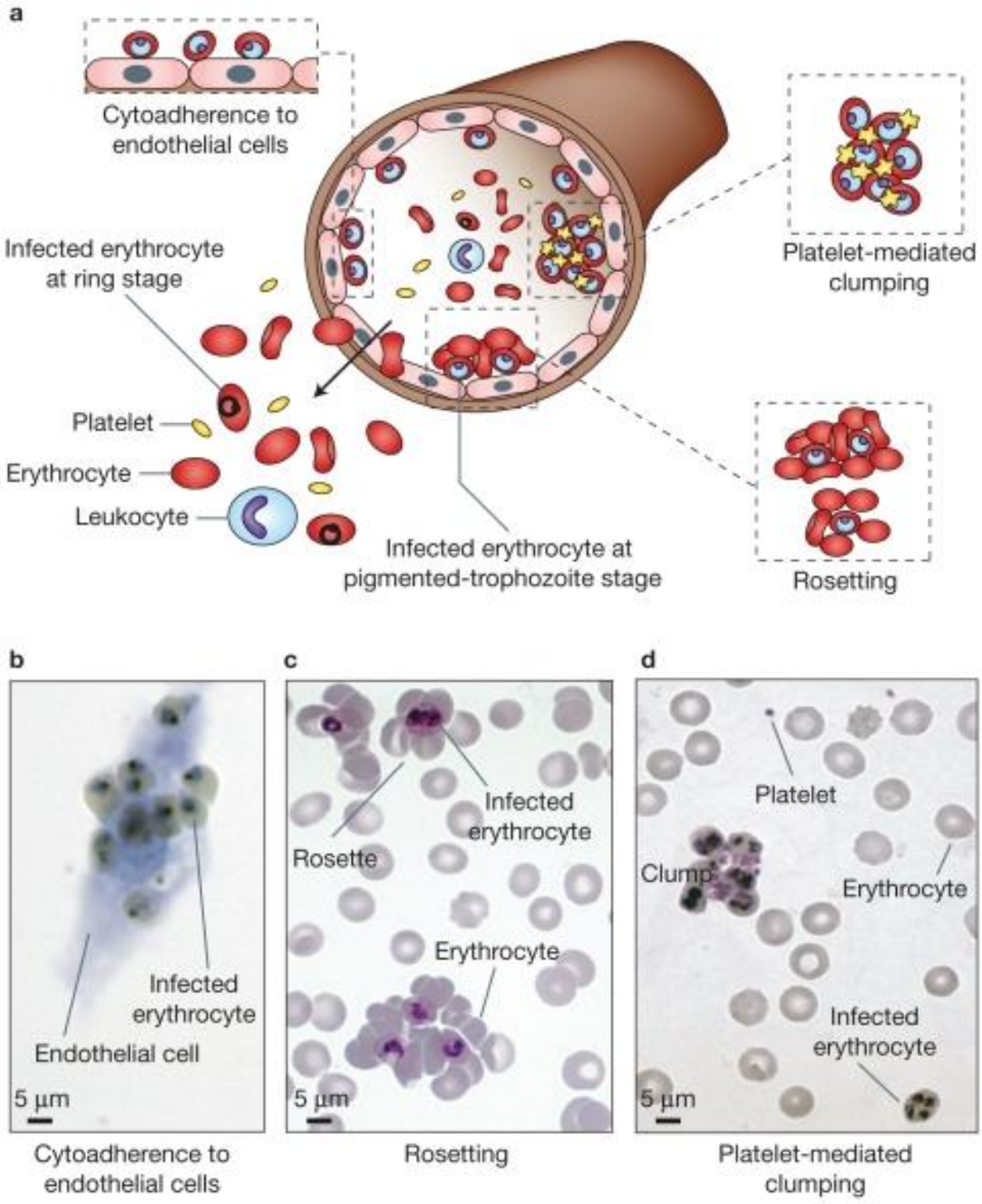


Figure 1.14. Cytoadhesion of erythrocytes with *P. falciparum* to human cell. *(From Expert Rev Mol Med 2009 26(11) e16)*

A novel mechanism that supports pRBC adhesion *in vitro* is platelet-decorated von Willebrand factor (vWF). vWF is a glycoprotein constitutively produced by the Weibel-Palade bodies in the endothelium and megakaryocytes present in the blood plasma that is important for blood clotting. Liberation of vWF multimers tethered to ECs into circulation is triggered by EC activation brought by inflammation responses. During *P. falciparum* infection, vWF levels are elevated in both CM and UM patients (Phiri, Bridges et al. 2011), which unravel under flow and accumulate platelets on their surface by binding to platelet receptors such as thrombin. The vWF is cleaved and regulated by protease ADAMTS 13 that has significantly reduced activity during malaria infection. The accumulation of platelets offers the opportunity for increased pRBC adhesion, thus permitting vWF to play a role in malaria pathogenesis (Bridges, Bunn et al. 2010).

Other receptors shown to mediate clumping are: P-selectin, expressed on the surface of activated platelets, especially in combination with CD36 (Wassmer, Taylor et al. 2008) and globular C1q receptor (gC1qR-HABP1-p32), expressed on the surface of diverse cell types including endothelial cells and activated platelets. gCIqR-HABP1-p32 has been shown to facilitate pRBC-platelet clumping as well as potentially acting as a direct cytoadherence receptor through expression on brain endothelia (Biswas, Hafiz et al. 2007). The parasite ligands that interact with P-selectin and gC1qR-HABP1-p32 have not yet been identified.

1.11 Regulation and control of *var* gene expression

Knowledge of the molecular mechanisms regulating antigenic variation has advanced considerably; however, some inconsistencies and unanswered questions remain. There are different schools of thought with regard to control of *var* gene expression. Reverse transcription (RT)–PCR studies indicate the presence of multiple *var* transcripts in ring-stage parasites, but only a single *var* transcript in mature trophozoites. This has led to some groups believing that several *var* genes are transcribed during ring stages and only one *var* gene is expressed in mature stages such as trophozoites, from approximately 16 hours post-invasion. This has been accredited to the detection of only one full-length mRNA message in these mature stages (Chen, Fernandez et al. 1998; Scherf, Hernandez-Rivas et al. 1998; Kyes, Christodoulou et al. 2007). Several lines of evidence have emerged that contradict this model of relaxed transcription of all *var* followed by exclusive transcription of a single *var*. This raised the question of a possibility of complete transcription of many *var* genes in trophozoite stages with a rapid and selective 3' to 5' degradation of the products not predestined for expression (Taylor, Kyes et al. 2000). This theory is backed-up by the analysis on synchronised mature parasites selected for adhesion to a certain receptor which has shown the detection of multiple full-length *var* genes transcripts (Noviyanti, Brown et al. 2001; Duffy, Brown et al. 2002), although one might argue that these arise from cytoplasmic remnants of promiscuous ring stage transcription.

It has also been argued that a bias towards transcription of the gene dominant in the previous asexual erythrocyte life cycle results in the same single *var* evading

silencing in the mature trophozoite (Chen, Fernandez et al. 1998). The process of exclusive transcription might be reset to a default *var* following transmission through the mosquito (Peters, Fowler et al. 2002). Initially, Chen et al identified FCR3S1.2$_{var1}$ as the dominant transcript belonging to group A-*var* genes transcribed in rosetting trophozoites of FCR3s1.2 parasite strains (Chen, Barragan et al. 1998). However using three sets of primer-pairs generated for the amplification of unknown DBL1α-sequences lead to the identification of a different dominantly transcribed *var* gene (FCR3S1.2$_{var2}$) in the same strain (Albrecht, Moll et al. 2011). These two studies highlight the importance of using more in-depth approach in the design of degenerate primers for detection of group A1. Regardless of whether only one full-length as opposed to several full-length *var* mRNA messages are present in mature asexual blood stages, the fact still remains that there is clonal expression of PfEMP1 proteins displaying different adhesive phenotypes by individually infected erythrocytes. The association of phenotypes with binding to particular tissues or disease outcome in field studies is evidence for transcription initiation control of gene expression.

Investigations pertaining to the molecular basis for transcriptional regulation of the multicopy gene families of *P. falciparum,* with emphasis on the *var* gene family, have particularly centred on the roles that chromatin structure, subnuclear localisation and DNA regulatory elements play in *var* gene silencing, activation and mutually exclusive expression (reviewed in (Dzikowski, Templeton et al. 2006). The concepts likely also apply to the other multicopy gene families found in the malaria genome, such as the 2TM gene family members, of which little is known.

Antigenic variation in microorganisms is created by two general types of mechanisms: genetic events that involve gene recombination and/or mutations, and epigenetic mechanisms that affect the expression of a gene without altering its primary nucleotide sequence. However, for *P. falciparum var* genes, transcription occurs *in situ* and does not require specific gene shuffling, deletions or altering the DNA sequence of its regulatory elements (Kyes, Horrocks et al. 2001) as is the case in other organisms (Scherf, Hernandez-Rivas et al. 1998). These results suggest that *var* gene activation or repression occurs under epigenetic control. Whether antigen variation is governed by genetics or epigenetics, the studies that have performed thorough investigations on the subject generally point towards the fact that antigenic variation is not random but occurs with a certain order at specific loci. Earlier work by (Scherf, Hernandez-Rivas et al. 1998; Deitsch, del Pinal et al. 1999) supported the idea that switching between expressed PfEMP1 forms is controlled by epigenetic mechanisms rather than genetic rearrangements as shown by (Smith, Chitnis et al. 1995). *In vitro* studies with *P. falciparum* isolates verified that the rate of antigenic switching of a certain variant was estimated as high as 2% per generation, with immune selection presumably restricting the expressed PfEMP1 repertoire *in vivo* (Biggs, Gooze et al. 1991; Roberts, Craig et al. 1992). In other words, at the end of each asexual erythrocytic cycle, 98% of schizonts express the original PfEMP1 type in the newly infected erythrocyte.

The molecular mechanisms for *var* switching remain elusive. Some data have suggested that regulation of *var* genes consists of 2 activities: interaction of the 5' promoter and the intron promoter, and chromatin modification, specifically histone

methylation and deacytelation (Deitsch, Calderwood et al. 2001; Calderwood, Gannoun-Zaki et al. 2003), and yet the mechanism underlying this interaction is unclear. Silent genes are characterised by a specific methylation of histone H3, H3K9me3 (Chookajorn, Dzikowski et al. 2007; Lopez-Rubio, Gontijo et al. 2007) whereas active genes are associated with the presence of H3K4me2 and H3K4me3. This selective modification of histones is believed to be involved in the highly structured *var* switching described by Recker et al (Recker, Buckee et al. 2011).

One of the major draw backs of using *in vitro* methods to study *var* transcription is the possible bias in transcription profiles due to long term cultures. Recent studies clearly demonstrate that even after few cycles in a short term culture, the expression profile of *var* genes can change rapidly (Peters, Fowler et al. 2007). To overcome these limitations elaborate bioinformatics analyses are required where identification of *var* sequences can be achieved by special algorithms of mere tags of semi-conserved domains like the DBL1α (Normark, Nilsson et al. 2007). In spite of this, the *var* regulation studies have been consistent in identifying the order governed by *var* switching. Thus, long term cultures in the absence of selective pressure rapidly result in extremely heterogenous *var* expression. Horrocks *et al*. were the first to show that *var* switching rates vary between isolates and are controlled by the individual genes intrinsic factor (Horrocks, Pinches et al. 2004). These rates were later proposed to be associated with chromosomal location, with *var* genes located in the centromere being stable and rarely undergoing transcriptional switches with low off rates ranging from 0-0.3% per generation, while *var* genes in the subtelomere readily switch with high off

rates 1-2% (Frank, Dzikowski et al. 2007). Mathematical models also predict substantially lower rates (0.03% per generation) of some variants (Paget-McNicol, Gatton et al. 2002) or faster (18%) for others (Gatton, Peters et al. 2003).

Recent models by Recker et al have shown that a non-random highly structured switching pathway utilises the different on and off rates of the variants to prevent exposure of the entire *var* repertoire to the host immune system during the early stages of infection (Recker, Buckee et al. 2011). In order for *P. falciparum* to limit antigen exposure to the host immunity, the variant switching needs to occur often enough to generate parasite subpopulations that escape the host immune response and at the same time tightly controlled in order to avoid premature expenditure of the 60 different proteins. Wang *et al*. have shown that parasites isolated from non-immune individuals express all *var* genes and let growth advantages select for those parasites best adapted to their hosts adhesion surfaces at onset of infection (Wang, Hermsen et al. 2009), as is the case in pregnancy malaria where the dominant variant in circulation is different from the parasite population sequestering in the placenta. .

The general control of *var* gene transcription is governed by two promoters (Figure 1.9A), one located upstream and regulated *var* expression; the second promoter is on the introns and is responsible for sterile transcripts. The same promoter was also shown to function in both directions (Calderwood, Gannoun-Zaki et al. 2003), hence the prospect that its anti-sense transcripts may play a role in *var2csar* regulation in CSA-selected parasites (Ralph, Bischoff et al. 2005). Widespread antisense transcripts are also believed to be involved in the modulation of gene expression in humans (Yelin,

Dahary et al. 2003), rice (Osato, Yamada et al. 2003; Yamada, Lim et al. 2003; Yelin, Dahary et al. 2003), and *Arabidopsis* (Yamada, Lim et al. 2003). Non-protein coding RNA is also reported to play a role antigenic variation and potentially regulate *P. falciparum* gene expression at all levels (Raabe, Sanchez et al. 2010).

1.12 Human acquired immunity and antibody reactivity to *Plasmodium falciparum*

1.12.1 Memory B cells

In theory, immunity to malaria parasite could be directed at any point in the life cycle, from the time the mosquito injects the sporozoites into the skin, to the pre-erythrocytic and erythrocytic stages (reviewed in (Langhorne, Ndungu et al. 2008)). Mouse models have demonstrated killing of the pre-erythrocytic stages in the hepatocytes using CD8+ effect cells producing IFN-γ (Schofield, Villaquiran et al. 1987). However some longitudinal studies have disputed these findings suggesting that immunity involvement of these stages is limited (Owusu-Agyei, Koram et al. 2001). During blood stages, humoral response is presumed to play a key role as shown by mouse models, which demonstrate parasite clearance at this stage of infection is mediated by antibodies, B cells (Langhorne, Cross et al. 1998) and T-cell-mediated release of IFN-γ from macrophages (Pombo, Lawrence et al. 2002).

People exposed to malaria do accumulate memory B cell specific for malaria antigens with exposure (Langhorne, Ndungu et al. 2008; Weiss, Traore et al. 2010; Wipasa, Suphavilai et al. 2010; Nduati, Gwela et al. 2011), however, studies investigating malaria-specific memory B cells in malaria-exposed people are limited.

One study reported a persistence of anti-*P. falciparum* memory B cells for over 8 years in adults without evident re-exposure (Migot, Chougnet et al. 1993) while another study reported low memory B cell in malaria-exposed children (Dorfman, Bejon et al. 2005). In addition, children and adults living in malaria-endemic areas have shown altered distribution of B-cell subset (Asito, Moormann et al. 2008; Weiss, Traore et al. 2010), suggesting some dysfunction of the B-cell compartment during acute and chronic *P. falciparum* malaria infection.

1.12.2 T-cells

Memory CD4+ T cells are also thought to be important in regulating pathology through cytokine production and inducing rapid secondary antibody responses (Langhorne, Ndungu et al. 2008). During malaria infections, memory CD4+ T cells are induced naturally, however, it has been difficult to demonstrate the function of CD4+ T cells in natural human infection due to low frequencies and the associated short-lived memory and regulatory activity (Langhorne, Ndungu et al. 2008; Todryk, Walther et al. 2009). Currently, very little is known about CD4+ T-cells on parasite surface antigens such as PfEMP-1. One study used DBLα-tags to investigate CD4+ T-cell response to PfEMP-1 dominantly expressed in children with SM and UM during acute infection and found antigen-type dependent T-cell responses difference between *var* group A PfEMP1 and non-group A *var*/PfEMP-1 (Gitau, Tuju et al. 2012).

1.12.3 Dendritic Cells

At the centre of both the T-cells and B cells responses are the dendritic cells (DC), which initiate and regulated the adaptive immune response. DCs specialize in the uptake, processing and presentation of antigens to T cells. Malaria parasites can impair and suppress DC function, thus enabling parasite to invade the immune system (reviewed in (Wykes, Keighley et al. 2007; Bousheri and Cao 2008; Todryk and Urban 2008)). DCs activate and mature during *Plasmodium* infection (Coban, Ishii et al. 2002; Leisewitz, Rockett et al. 2004; Sponaas, Cadman et al. 2006; Wilson, Behrens et al. 2006) however, their activity is suppressed by haemozoin, a by-product of the malaria infection (Skorokhod, Alessio et al. 2004; Millington, Di Lorenzo et al. 2006) resulting in unstable interactions with T-cell (Millington, Gibson et al. 2007).

1.12.4 Antibodies

Immunity to malaria is acquired as a result of antigenic stimulation through repeated infections from early childhood onwards (McGregor 1987). This concept is demonstrated by the fact that in areas where malaria is endemic, the age-specific burden of *P. falciparum* infection and clinical disease are closely related to the level of malaria transmission. In high-transmission areas, the youngest children suffer from frequent episodes of disease and high parasite loads, while older individuals are generally able to control parasitemia and only suffer from mild malaria symptoms. On the contrary, in low-transmission areas, the incidence and severity of clinical disease in adults remains similar to that of children (Snow, Bastos de Azevedo et al. 1994; Snow,

Omumbo et al. 1997; Greenwood and Mutabingwa 2002; Lusingu, Vestergaard et al. 2004).

Immunoglobulin G antibodies (IgG) are among the immune responses associated with protection against clinical malaria, including antibodies with specificity for variant surface antigens (VSA) expressed on the surface of pRBC (Marsh, Otoo et al. 1989; Bull, Lowe et al. 1998; Giha, Staalsoe et al. 2000; Bull and Marsh 2002). *P. falciparum* can maintain a chronic infection despite the sequential immune clearance of phenotypically similar parasites, due to emergence of isogenic parasite populations expressing different variant antigens on the surface of the pRBC that are not recognised by the host immune system. Accumulated clinical immunity development coincides with the gradual acquisition of a broad repertoire of VSA-specific antibodies (Bull, Lowe et al. 1998; Hviid and Staalsoe 2004). An infection is accompanied by development of antibodies with specificity for the VSA expressed by the infecting parasite (Marsh, Otoo et al. 1989; Ofori, Dodoo et al. 2002; Staalsoe, Shulman et al. 2004; Elliott, Brennan et al. 2005). This process is repeated following each new parasite infection and it appears to protect the host from future clinical episodes arising from parasites expressing antigenically similar VSA. Antibodies are effective presumably by blocking invasion of RBC by merozoites (Blackman, Heidrich et al. 1990) cellular killing of antibody-opsonised parasites (Bouharoun-Tayoun, Oeuvray et al. 1995) and/or binding of antibodied to parasite-induced molecules (Bull, Lowe et al. 1998).

VSA expressed by parasites isolated from children with severe disease have been found to be more frequently recognised by plasma from the same (or different)

patient taken during and after infection in areas of moderate to high transmission (Bull, Lowe et al. 1999; Bull, Kortok et al. 2000; Nielsen, Staalsoe et al. 2002). Such events occur due to the antibodies ability to cross-react by recognising the same epitopes. Following these observations, it was later discovered that in high transmission areas, young children (who are more prone to severe disease) quickly acquire antibodies and protection against malaria parasites expressing VSA types associated with severe disease outcomes.

The recent identification of specific VSA mediating parasite adherence to human brain endothelia (Avril, Tripathi et al. 2012; Claessens, Adams et al. 2012; Lavstsen, Turner et al. 2012) gives hope of a vaccine targeting SM-specific VSA. Parasite virulence may be linked to the expression of specific VSA that allows particularly effective pRBC sequestration through simultaneous interaction with several host receptors or strong and efficient adhesion to a single receptor (Chen, Heddini et al. 2000; Heddini, Pettersson et al. 2001). The necessity to express multiple adhesive domains ought to impose considerable constraints on the three-dimension structure of the VSA involved, suggesting that such VSA may intrinsically be more similar to each other (Nielsen, Vestergaard et al. 2004). Although extensive sequence diversity of VSA between and within clones has been demonstrated by several studies, it does not necessarily translate into serological diversity (Nielsen, Vestergaard et al. 2004).

Agglutination assays have been successful in describing antigenic variation in PfEMP1. Previously, agglutination studies have shown that antibodies from acute or convalescent patients agglutinate with *P. knowlesi* (Brown and Brown 1965) and *P.*

falciparum (Newbold, Pinches et al. 1992) in a variant-specific manner, and switches in agglutinating phenotype have been correlated with switches in cytoadherence characteristics of the pRBC (Roberts, Craig et al. 1992).

Surveys of agglutinating antibody responses to natural *P. falciparum* populations in Pakistan (Iqbal, Perlmann et al. 1993), The Gambia, (Marsh and Howard 1986) Kenya (Bull, Lowe et al. 1999) and Papua New Guinea (Forsyth, Philip et al. 1989; Reeder, Rogerson et al. 1994) have indicated that PfEMP1 antigens are highly diverse since antibodies induced following an infection generally agglutinate only the homologous parasite isolate that caused that particular infection. These findings confirm VSA diversity and implicate these molecules as important targets for naturally acquired immunity.

Pre-existing anti-PfEMP1 antibodies are passed from mother to child and last for about six months. These antibodies are able to recognise PfEMP1 variants that are expressed during a new infection of the infant and a subset of variants in the population. These results are supported by Bull et al. 1999 who found that plasma from a 3 month old child, presumably still carrying maternal antibodies, was able to agglutinate all isolates at the time of disease, apart from the homologous isolate. This gave rise to the concept that infection is due to a hole in the antibody repertoire. Despite the apparent role of anti-PfEMP1 antibodies in the development of anti-disease immunity, their diversity is thought to limit their potential as vaccine candidates. Although the total pool of PfEMP1 epitopes is generally assumed to be large, Aguiar and co-workers have argued that antigenic diversity appears to be finite

because of the fact that semi-immune serum has been found to agglutinate parasites isolated in different continents and those isolated from a similar location up to 19 years in the past (Aguiar, Albrecht et al. 1992).

Hviid et al propose that the specificity/cross-reactivity balance is determined by transmission intensity through its impact on acquisition of protective immunity (Hviid, Staalsoe et al. 2003). After all, it has been shown that antibody recognition of VSA varies markedly between different parasite isolates (Bull, Lowe et al. 1999; Bull, Kortok et al. 2000; Nielsen, Staalsoe et al. 2002), with parasites expressing common VSA at a selective advantage in non-immune patients but the balance gradually tips in favour of rare VSA with the acquisition of protective immunity. This hypothesis helps to solve the long-standing but unresolved puzzle of why immunity to severe disease develops much more rapidly than immunity to parasitaemia per se. If the common VSA are more conserved (and cross-reactive) among isolates than rare VSA, then the differences between the above geographical data from India and Sudan on one hand and the Gambia, Ghana, and Kenya on the other, probably reflect differences in seasonality and intensity of malaria transmission.

The *var* gene group A are highlighted as potential virulent PfEMP1 candidates for vaccines that can induce antibodies to inhibit rosetting (Discussed in section 1.10) and prevent severe malaria. The link between rosetting, group A *vars* and SM has been demonstrated *in vitro* where parasites selected to bind human brain endothelial cell line HBEC-5i consistently up-regulated group A-like *var* genes (Claessens, Adams et al.

2012). Further work from the same group links IgM-positive rosetting parasites and group A *vars*, showing that the rosetting group A type PfEMP1 have a similar head structure, with a distinct NTS (Ghumra, Semblat et al. 2012). Combinations of antibodies raised against the NTS segment and the various domains recognised live IEs (Ghumra, Khunrae et al. 2011) and blocked binding to HBEC-5i (Claessens, Adams et al. 2012), inhibited rosette formation and induced phagocytosis of pRBCs against homologous parasites (Barfod, Dalgaard et al. 2011; Ghumra, Khunrae et al. 2011; Ghumra, Semblat et al. 2012). Furthermore, the antibodies displayed broad cross-reactivity against heterologous parasite strains with the same rosetting phenotype, and clinical isolates from other sub-Saharan African countries (Ghumra, Semblat et al. 2012). The studies described above provide some evidence that parasites with a virulence-associated adhesion phenotype share pRBC surface epitopes that can be targeted by strain-transcending antibodies to PfEMP1, which potentially can be used in development of therapeutic interventions to prevent severe malaria.

1.13 Vaccines

Some high transmission areas in sub-Saharan Africa have experienced a significant reduction in malaria due to several efforts that have been put in place to control the disease. However, this success story is being threatened by the potential spread of artemisinin resistant strains of *P. falciparum* originating from Southeast Asia (White 2010) and the spread of *An. gambiae* strains that are resistant to pyrethroid

insecticides (WHO 2011). It is through such challenges that the idea of a vaccine seems essential, especially in areas where elimination is inconceivable.

1.13.1 Pre-erythrocyte vaccines

A vaccine targeting the sporozoite is very logical because only a small number of parasites (Sinden 2010)are inoculated at a time, thus creating a bottleneck in the parasite life cycle. The idea of designing a vaccine to target pre-erythrocytic stages started when UV-irradiated sporozoites of *P. gallinaceum* showed protection from avian malaria in chickens (Ceithaml and Evans 1946). Subsequently, vaccination of rodents with irradiated sporozoites of *P. berghei* showed maximum protection against challenge (Nussenzweig, Vanderberg et al. 1967). Since the identification of the sporozoite surface molecule circumsporozoite protein (CSP) as a target for antibodies (Sinden 2010), several efforts have used the CSP for vaccine development with some success in humans (Vanderberg, Nussenzweig et al. 1969; Clyde, Most et al. 1973; Patarroyo, Amador et al. 1988). The required amount of attenuated sporozoites to elicit a response have been recently quantified, therefore it is hoped that clinical trials will proceed shortly (Hoffman, Billingsley et al. 2010). Other protective mechanisms in humans have been achieved by exposure to *P. falciparum*-infected mosquitoes followed by treatment with chloroquine (Roestenberg, Teirlinck et al. 2011).

There is a potential synthetic vaccine RTS,S, made from CSP, with hepatitis B virus surface antigen and an adjuvant AS02 and AS01 (monophosphoryl lipid A , quil A and saponin or liposomes) (Casares, Brumeanu et al. 2010) which shows 30-50%

protection and that lasts longer than four years in some cases while in others, RTS,S has failed to elicit high enough immune response to some antigens (Casares, Brumeanu et al. 2010; Greenwood and Targett 2011; Garcia-Basteiro, Bassat et al. 2012). Phase 3 trials of this vaccine are currently underway at eleven sites in seven countries, including Malawi.

1.13.2 Blood stage vaccine

Efforts in blood stage vaccines to block erythrocyte invasion or inhibit parasite replication have mostly been based on the following antigens: the apical membrane protein 1 (AMA-1), MSP1, 2 and 3, GLURP, RESA, serine repeat antigen 5 (SERA5) and the erythrocyte-binding antigen 175 (EBA-175) (WHO 2010) with minimal success in humans (Genton, Betuela et al. 2002; Ellis, Sagara et al. 2010; WHO 2010; Thera, Doumbo et al. 2011). In other attempts, infections induced by bouts of *P. falciparum* erythrocytes that are treated before manifestation of the erythrocytic stages show protection from subsequent challenge and elicit a strong T-cell proliferation (McCarthy and Good 2010).

1.13.3 Vaccines that impact transmission

Other vaccines designed to control transmission, P320, P48/45 and HAP2, have been based on gametocyte surface antigens and prevent fertilisation in the mosquito midgut. Alternatively, P25 and P28 vaccines induce antibodies that prevent zygote or ookinete migration from the midgut (Arevalo-Herrera, Solarte et al. 2011). Only P25,

based on the ookinete antigens of *P. falciparum* and *P. vivax,* has reached the clinical phase of development (Malkin, Durbin et al. 2005; Wu, Ellis et al. 2008; Arevalo-Herrera, Chitnis et al. 2010; Arevalo-Herrera, Solarte et al. 2011) but no large clinical trials have yet been undertaken.

1.13.4 PfEMP1 based-vaccines

Variant specific anti-PfEMP1 antibodies are thought to contribute to the regulation of parasite density in a manner that decreases the incidence of clinical disease (Marsh and Howard 1986; Forsyth, Philip et al. 1989; Gupta, Hill et al. 1994; Bull, Lowe et al. 1998). As previously discussed in section 1.12.4, immunity to PfEMP1 can thereby influence transmission by regulating the density of asexual blood stages with the potential to become transmission stages, and to directly target early gametocytes to prevent their maturation into transmission stages (Piper, Roberts et al. 1999). On the other hand vaccine against variant-specific PfEMP1 epitopes might be unrealistic due to complex variability in these antigens but there is some hope as selected PfEMP1 variants have been associated with severe malaria. Therefore, subgroups of these antigens, such as the *var*2csa important in PM have the potential to make good vaccine candidates for specific aspects of the disease (Hviid 2010; Avril, Cartwright et al. 2011). Alternatively, targeting the relatively conserved DBL1 domain to generate cross-reacting antibodies may also circumvent the problem of variant PfEMP1 (reviewed in (Ho and White 1999)).

The CIDR1 domain of PfEMP1 is highly variable (Smith, Subramanian et al. 2000). Although the CIDR1 domain was unable to elicit high antibody titers during infection (Baruch, Gormely et al. 1996; Baruch, Ma et al. 1997), monoclonal antibodies produced against different regions of this domain have reacted with several isolates (90% of those tested) expressing different PfEMP1 variants except those that do not bind to CD36 (Gamain, Smith et al. 2001), presumably because the monoclonal antibodies were raised to a CIDRα and not a CIDRα_1 type domain. Some immunisation experiments in monkeys using a 179-amino acid region of the CIDR domain induced protection against homologous challenge (Baruch, Gamain et al. 2002). Furthermore, immunisation of mice with three different CIDR1 that have the critical function of binding to CD36 also induced cross-reactive antibodies (Gratepanche, Gamain et al. 2003). Thus, CIDR1α-based general malaria vaccines have served the proof-of-principle of the possibilities of developing vaccines against variant antigens and other PfEMP1 domains are also being exploited for this purpose (Chen, Pettersson et al. 2004).

1.14 *Var* gene studies in field isolates

Advances in the study of *var* gene expression in field isolates have been made due to the availability of robust molecular techniques which have allowed the determination of various surface antigens expressed on the surface of an infected erythrocyte in relation to cytoadherence and disease severity. A highly dynamic and variable picture of simultaneously expressed *var* transcripts has been observed in field isolate samples confirming the recombinogenic nature of *var* genes, with a correlation

between the number of *var* transcripts and the number of infecting strains (Kaestli, Cortes et al. 2004; Kaestli, Cockburn et al. 2006). This has raised a common challenge of identifying several non-identical sequences between different isolates. This complication poses challenges in how the enormous amount of data that has already been generated can be meticulously analysed and fully understood. This problem is well pointed out by (Barry, Leliwa-Sytek et al. 2007) who observed a vast diversity of DBL1α domains of *var* genes in the genome of parasites sampled from widespread geographic origins compared to parasites from a single malaria endemic area of Papua New Guinea (PNG).

The vast *var* gene diversity is largely responsible for the inherent difficulties in population genomic analysis of highly diverse multigene families of *Plasmodium spp*. Individual *P. falciparum* genomes have repertoires of *var* genes that can recombine with other repertoires during the sexual phase of the life cycle in the mosquito (Su, Ferdig et al. 1999). There is also circumstantial evidence for ectopic recombination among *var* genes within the same genome, possibly during both meiosis and mitosis, creating a possibility to generate diversity even among closely related genomes (Ward, Clottey et al. 1999; Freitas-Junior, Bottius et al. 2000; Taylor, Kyes et al. 2000). Additional complications in the *P. falciparum* genome are the several unusual features such as extreme AT bias, large tracts of non-unique sequences and several large families of polymorphic genes (Gardner, Hall et al. 2002).

Parasite populations are generally distinct based on geographical location. For example, South American and Asia-Pacific isolates commonly amplified identical DBL1α

sequences from multiple patients, whereas this is a rare occurrence in sub-Saharan African samplings of circulating populations (Nogueira, Wunderlich et al. 2001; Fowler, Peters et al. 2002; Bull, Berriman et al. 2005; Albrecht, Merino et al. 2006; Fowler, Chavchich et al. 2006; Barry, Leliwa-Sytek et al. 2007). Bull *et al.* have shown that *var* genes from Kenyan field isolates and laboratory isolates can be classified into biologically meaningful subsets based on cysteine-containing small blocks of semi-conserved sequences, such as the DBL domain cassette classification described by Lavstsen *et al* (Rask, Hansen et al. 2010) thus providing some evidence of the existence of *var* gene semi-structuring (Bull, Berriman et al. 2005). However, there was variation in expression of these semi-conserved sequences in parasites. In most of the isolates there were clear dominant sequences present at high frequencies expressed by the parasite in different infections that were consistent with those found in field isolates of other studies. Further, Warimwe *et al*. from the same group showed that expression of a minor component of all genomic *var* repertoires of semi-conserved blocks with two cysteine residues, "cys2", were associated with parasites from young children with severe malaria and low immunity against malaria (Warimwe, Keane et al. 2009). The *var* genes with the 2 cysteine structure mostly belong to group A *var* genes. Therefore, the results are compatible with the hypothesis that the genomic *var* gene repertoire is organised such that PfEMP1 molecules that confer the most virulence to the parasite belong to group A *vars* (Kyriacou, Stone et al. 2006).

So far, *var* gene studies have employed the use of polymerase chain reaction (PCR) techniques for the amplification of parasite DNA. A conserved DBL1α domain is

targeted using “universal” primers to amplify a tag of variable sequence and length with which to identify each transcript distinct. These primers were subsequently shown to exhibit bias for a subset of *var* genes, affecting sequence abundances by leaving other *var* sequences untargeted (Taylor, Kyes et al. 2000). A new set of universal primers designed by Kyes *et al.* in 1997, revised by Taylor in 2000 and again by Bull in 2005, are the most utilised for field studies of all the primer sets developed so far (Kyes, Taylor et al. 1997; Taylor, Kyes et al. 2000; Bull, Pain et al. 2005).

Studies on circulating parasites have demonstrated that there is high turnover in the antigens expressed during infection with most studies linking high expression of group A *var* genes in peripheral blood to severe disease syndromes (Bull, Berriman et al. 2005; Kaestli, Cockburn et al. 2006; Kyriacou, Stone et al. 2006; Rottmann, Lavstsen et al. 2006; Warimwe, Keane et al. 2009). Examination of postmortem samples of severe infections has shown a reduced genetic diversity compared to mild and asymptomatic paediatric malaria (Montgomery, Milner Jr. et al. 2006; Milner, Valim et al. 2012). Their pattern of antigen expression is, however, largely unknown. Nonetheless, Dobano *et al* used immunofluorescence antibody typing for MSP 1 and 2 alleles to compare circulating parasites and sequestered parasites in the brain and other tissues in the same Malawian children with fatal malaria (Dobano, Rogerson et al. 2007). They found concordance between parasite serotypes in peripheral blood and parasite serotypes in tissues and no difference in the serotype distributions in the different tissues. Previous studies of sequestered parasites have utilised purified receptors or cultured endothelium from particular organs, plus clinical isolates and

laboratory-adapted lines selected for specific adhesive behaviour, to model and investigate potential host-parasite interactions (Gay, Robert et al. 1995; Prudhomme, Sherman et al. 1996; Xiao, Yang et al. 1996; Traore, Muanza et al. 2000).

There is evidence that the inflammatory responses seen at the blood-brain barrier in cases of CM involves the increased systematic production of pro-inflammatory cytokines such as TNF, lymphotoxin, IFN -γ and IL1β (Kwiatkowski, Hill et al. 1990; Newton and Krishna 1998; Brown, Turner et al. 1999) induced by the immune response to the malaria parasites. The overproduction of cytokines such as TNF results in up-regulation on cerebral EC of ICAM-1, VCAM-1 and E-selectin that facilitates the sticking of pRBC to host receptors when they are expressing the appropriate ligands (Ockenhouse, Ho et al. 1991; Turner, Morrison et al. 1994; Rogerson, Tembenu et al. 1999; Silamut, Phu et al. 1999). In accordance with this, a recent study on paediatric malaria in Ghana also showed staining for ICAM-1, VCAM-1 and E-selectin in association with pRBC in brain microvasculature of fatal CM patients (Armah, Dodoo et al. 2005). However, the specific ligands used by the pRBC during *in vivo* interactions are unknown, largely due to the immense diversity and complexity of these antigens, and the inaccessibility of sequestered parasites.

As previously mentioned, several adhesive phenotypes that do not utilise endothelia such as rosetting, sequestration of pRBC in the placenta (Miller, Baruch et al. 2002), formation of platelet-mediated clumps (Pain, Ferguson et al. 2001), and pRBC adhesion to vWF (Bridges, Bunn et al. 2010) are all thought to contribute to pathology. Such pRBC adhesion mechanisms are supported by post-mortem histological studies

that have shown pRBC accumulating in the microvasculature (Grau, Mackenzie et al. 2003; Wassmer, Combes et al. 2006).

Malawi has a high burden of malaria with no reduction in disease incidence observed over the past decade despite a change of first-line anti-malarial treatment in 2007 and intensification of vector control programmes (Roca-Feltrer, Kwizombe et al. 2012). As such, there is a local malaria surveillance at the QECH and the paediatric clinicopathological study of fatal malaria patients in Blantyre, Malawi, with more than 100 study cases of fatal malaria, including controls, from 1996-2011. The availability of such resources has allowed investigation of host-parasite clumping mechanism and examination of antigen properties of sequestered populations of pRBC in the brain, heart and gut and their interactions with the host.

PROJECT AIMS

The purpose of this work is to study tissue samples from a malaria clinicopathology study of fatal pediatric malaria patients. The main aims of the study are to:

1. Determine which *var* gene groups are expressed by parasites in severe malaria and sequestered in cerebral microvasculature
2. Determine the expression of putative sequestration receptors and cytokines in different paediatric malaria diagnostic groups and organs from fatal cases of *P. falciparum* paediatric malaria
3. Determine which particular *var*/PfEMP1 subtypes and ABO blood groups mediate platelet-mediating clumping

Chapter 2

2. STUDY PARTICIPANTS, MATERIALS AND METHODS

2.1 Study location

2.1.1 Malawi

The republic of Malawi is a landlocked country located in south-east Africa, bordered by Zambia in the west, Tanzania in the north-east and Mozambique on the south-west and east. Much of the country is separated from Tanzania and Mozambique by the physical barrier of Lake Malawi. Malawi covers over 118,000 km^2 (45,560 sq mi) with an estimated population of more than 15.3 million as of 2009 (http://www.who.int/countries/mwi/en/). The country is divided into three main regions: Northern, Central and Southern Regions which are further sub-divided into a total of 24 districts comprising of several ethnic groups with English and Nyanja as the official languages. The largest and capital city is Lilongwe, located in the Central Region. The main commercial city of the country, Blantyre is located in the Southern Region.

Figure 2.1. Study location in Malawi.

Malawi is one of the least developed countries in the world with a Gross National Capita income of USD810 per annum. Agriculture is the main driving force of the economy, accounting for at least 90% of export revenue with tobacco being the main export crop. Malawi has a major burden of disease including HIV/AIDS, pneumonia, tuberculosis and diarrhoea with malaria being the leading cause of death in children under five years (http://www.who.int/countries/mwi/en/).

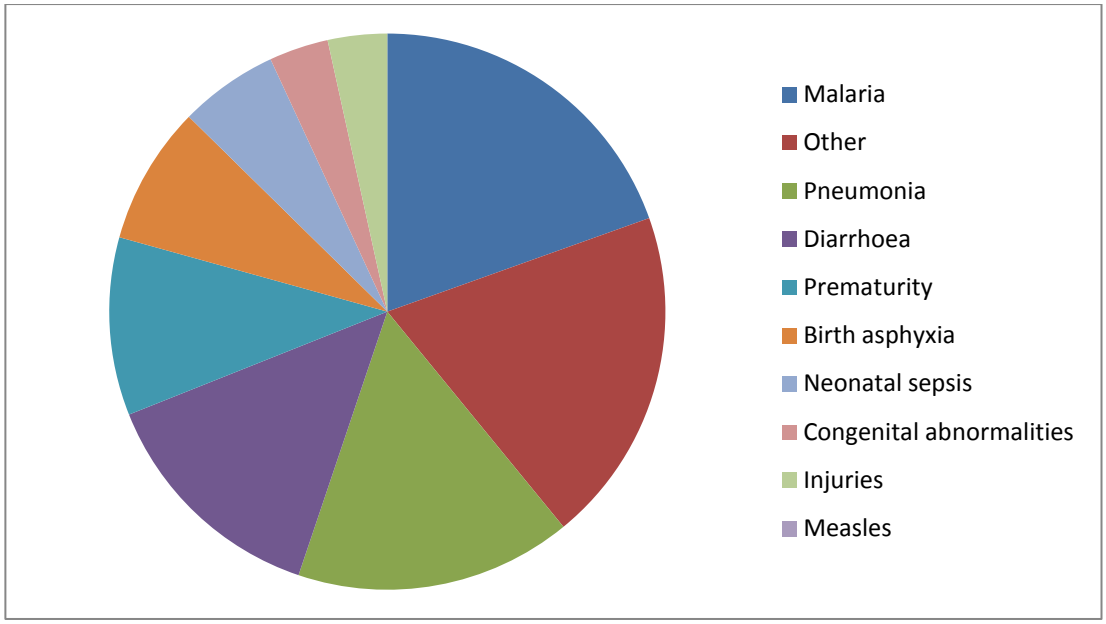


Figure 2.2. Distribution of causes of death in children less than five years.

These are figures from 2008 adapted from http://www.who.int/countries/mwi/en/ Malaria was listed as the main cause of under-5 death

2.1.2 Malawi-Liverpool-Wellcome Trust Clinical Research Programme

The Malawi-Liverpool-Wellcome Trust Clinical Research Programme (MLW), located in Blantyre, was established in 1995 for the purpose of conducting research on diseases of local importance and providing training for clinical and laboratory staff. The programme operates in collaboration with the College of Medicine, University of Malawi (COM) and the Liverpool School of Tropical Medicine and University of Liverpool, UK, via the Wellcome Trust Tropical Centre. Clinical research performed at MLW is based in the wards and clinics of the Queen Elizabeth Central Hospital (QECH) and in health centres in the peri-urban and rural areas.

QECH is a 1000 bed referral hospital for the Southern Region, is the largest hospital in Malawi and also serves as a local hospital for residents of Blantyre and surrounding areas, a population of 728,285 (www.world-gazetteer.com 2012). This study was conducted at the MLW laboratories which have successfully passed both internal and external quality control. Ethical approval was obtained from the College of Medicine Research and Ethics Committee (COMREC) under the University of Malawi, as detailed below. All clinical procedures were undertaken by experienced and Good Clinical and Laboratory Practice (GPLC) trained nurses and clinicians.

2.1.3 Malaria surveillance in Malawi

Malaria is responsible for approximately 40% of hospitalisation of children under the age of five (WHO report 2010). The Ministry of Health in collaboration with its partners implemented the National Malaria Strategic Plan from 2005–2010 with the goal to scale up malaria interventions towards the national vision of "Malaria-free Malawi." There were four main areas of focus: effective antimalarial treatment, ITNs, IRS and prevention of malaria in pregnancy (MIS report 2010).

Some of the highlighted interventions included a change in treatment policy from sulfadoxine-pyrimethamine (SP) to artemisinin-based combination therapy (ACT) in 2007 for *P. falciparum* infection. All severe cases are still treated with quinine. In pregnant women, malaria prevention relies both on the use of ITNs and IPT with SP for women during pregnancy (PMI report 2012; MIS report 2010).

The use of ITNs when sleeping is the primary control strategy for preventing malaria in Malawi. Results from the 2010 Malawi Malaria Indicator Survey (MIS) indicate that 58.2% of households possess at least one ITN and among these houses, 80.7% of children under the age of five slept under an ITN the night before the survey. ITNs are distributed freely to all age groups since 2010 (MIS report 2010).

According to the MIS 2010, although IRS was approved since 2007, it has not yet been fully implemented as a malaria prevention method. Initially, IRS activities were limited to private spraying in Blantyre city, with less than 2% of all households sprayed in a year and pilot IRS spraying was started in Nkhotakota district. Based on the success of these efforts (Skarbinski 2012), the Malawi Ministry of Health have expanded IRS to a total of 7 districts (discussed in Chapter 1 section 3.2.2).

2.2 Study population

2.2.1 Clinical samples

This study utilises clinical samples collected under a clinicopathological study of fatal paediatric malaria in Blantyre, Malawi, that aimed to collect samples from 100 patients and did so between 1996-2009 (Taylor, Fu et al. 2004). Patients were admitted to the paediatric research ward at QECH and were classified during life as having CM (Blantyre coma score of $\leqslant$2, peripheral *P. falciparum* parasitaemia, and no other identifiable cause of coma); SMA (peripheral *P. falciparum* parasitaemia, a hematocrit level of <15% at any time during hospitalization, and consciousness until $\leqslant$2 hrs before

death); or cerebral malaria and anemia (CM + SMA; symptoms of CM plus a hematocrit level of ⩽15%). These classifications were confirmed by autopsy.

The time between admission and death ranged between 15 ±8 hrs, and autopsies were performed approximately 8 ±4 hrs after death. Tissue samples were placed in either tissue matrix (OCT compound; Tissue-Tek) or RNAlater (Ambion) at the time of autopsy, snap frozen in liquid nitrogen and stored at −80°C. Tissue was also fixed in 10% buffered formalin and processed using routine paraffin embedding for histological examination. The clinicopathological study was reviewed and approved by COMREC and ethics committees at Michigan State University and the University of Liverpool.

This project used frozen archived samples from between January 1999 – December 2008. The fatal malaria cases were divided into three diagnostic groups which had clinically defined CM with the following histological findings:

1. CM2 – classic CM, with high parasite burden in cerebral microvasculature and associated pathology such as ring haemorrhages, thrombi and infiltration of monocytes
2. CM1 – clinically high parasite burden in the brain but with no associated pathology
3. PC – parasitaemic controls are children who died with a mild or asymptomatic *P. falciparum* infection but with another identified cause of death

All patients had their HIV status determined with two rapid tests; Uni-Gold (Trinity Biotech, Carlsbad, CA) and Determine (Inverness Medical, Orlando, Florida). In the event of a discordant HIV result a PCR was performed. CM2 accounted for approximately half of the cohort and a quarter each of CM1 and PC cases (Taylor, Fu et al. 2004). The project was reviewed and approved by COMREC (Ref: P.06/07/560 and P.02/10/867). Details of these patients are described in Chapter 3.

2.2.2 *P. falciparum* platelet-mediated clumping study

2.2.2.1 Blood sample collection

Paediatric malaria patients under the age of 12 years were recruited after obtaining informed consent from their parents or guardians at the accident and emergency (A&E) ward at QECH. The summary of the patient characteristics are given in Table 2.1 and the full details are listed in Table 2.2. The study was approved by COMREC (Ref: P.02/10/867).

3 mL of whole blood was collected via venepuncture into sodium citrate tubes (BD vacutainer, product number 366395) and processed immediately.

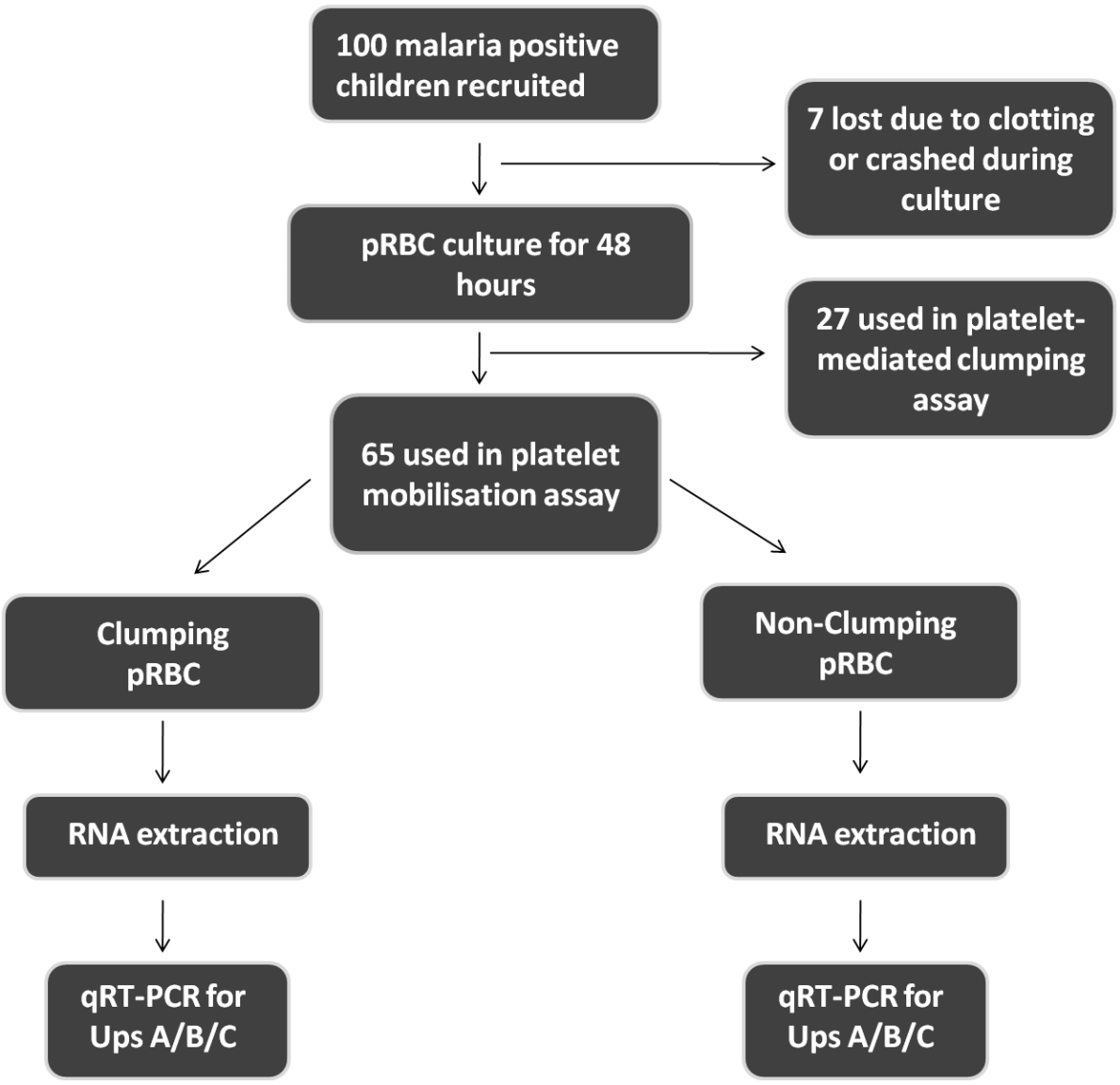


Figure 2.3. Flow chart of the *P. falciparum* platelet-mediated clumping study design. Infected erythrocytes were isolated from 100 *P. falciparum*-infected children and cultured to maturity. RNA was extracted from both the platelet-mediated clumping and non-clumping pRBC for determination of *var* gene group expression.

Table 2.1. Summary of the selected characteristics of the study population (n=100)

Characteristic	No.	(%)
Age in months		
Average	64	
Median	60	
Range	10-138	
Sex		
Female	48	58.9
Male	52	41.1
Blood group		
A+	17	23.2
B+	21	28.8
AB+	3	4.1
O+	21	28.8
O-	10	13.7
parasitaemia		
++	11	15.1
+++	17	23.3
++++	45	61.6

+	1 - 10 parasites per 100 thick film fields = approx 40 - 100/µL
+ +	11 - 100 parasites per 100 thick film fields = approx 100 – 1,000/µL
+ + +	1-10 parasites per single thick film field =approx 1,000 - 10,000/µL
+ + + +	> 10 parasites per single thick film field = approx 10,000-100,000/µL
+ + + + +	>100 parasites per single thick film field =>100,000/µL

Table 2.2. Clinical characteristics of patients in the study.

Sample No.	Age in months	Gender	Parasitaemia	PCV	Blood* group
1	36	Female	3+	18	
2	54	Female	3+	34	
3	96	Female	4+	21	
4	7	Female	4+	24	
5	18	Female	4+	30	
6	120	Male	2+	39	
7	78	Male	3+	22	
8	19	Female	4+	20	
9	97	Female	4+	30	
10	29	Female	2+	36	
11	93	Female	4+	35	
12	107	Male	4+	38	
13	84	Female	3+	-	
14	68	Female	2+	21	
15	32	Male	2+	22	
16	84	Female	3+	30	
17	108	Male	3+	-	
18	95	Female	3+	30	
19	67	Female	4+	21	
20	24	Male	4+	32	
21	60	Male	3+	22	
22	33	Male	2+	34	
23	55	Female	2+	27	
24	10	Female	4+	27	
25	53	Female	2+	28	
26	48	Male	2+	30	
27	48	Male	4+	38	A+
28	84	Male	4+	30	A+

29	132	Male	4+	30	O+
30	84	Male	4+	39	O+
31	108	Male	4+	40	O+
32	84	Male	4+	35	O+
33	84	Male	4+	40	A+
34	72	Male	4+	41	O+
35	72	Female	4+	32	O+
36	27	Male	4+	37	B+
37	132	Female	3+	39	O+
38	35	Female	3+	34	O+
39	78	Male	4+	37	B+
40	84	Male	4+	32	A+
41	25	Female	4+	30	AB+
42	96	Female	3+	30	B+
43	60	Male	4+	30	A+
44	132	Female	4+	38	A+
45	53	Female	4+	22	A+
46	53	Male	4+	25	A+
47	81	Female	4+	21	O-
48	48	Female	2+	26	B+
49	36	Male	4+	33	O-
50	43	Female	3+	20	A+
51	54	Female	3+	30	A+
52	108	Male	4+	41	O+
53	84	Male	3+	37	O-
54	72	Male	2+	39	O+
55	132	Female	4+	38	B+
56	60	Male	3+	33	AB+
57	17	Female	-	26	-
58	48	Female	3+	30	B+
59	108	Male	3+	36	A+

60	8	Female	2+	18	O-
61	96	Male	4+	29	B+
62	108	Male	4+	30	O-
63	60	Male	3+	32	B+
64	108	Male	4+		B+
65	108	Male	3+	45	B+
66	63	Female	4+	35	-
67	84	Male	4+	33	B+
68	96	Female	3+	35	O-
69	69	Male	2+	25	O-
70	96	Male	3+	35	O-
71	72	Male	4+	30	B+
72	30	Female	4+		O-
73	10	Female	3+	26	B+
74	44	Male	4+	36	B+
75	60	Female	4+	20	B+
76	138	Male	4+	31	O-
77	48	Female	2+	36	A+
78	15	Male	4+	36	B+
79	17	Female	4+	33	B+
80	18	Female	4+	23	A+
81	24	Female	4+	31	B+
82	23	Male	3+	28	O+
83	24	Male	4+	29	A+
84	36	Male	4+	27	O+
85	72	Female	2+	35	O+
86	24	Female	2+	30	O+
87	132	Female	4+	34	B+
88	26	Male	3+	28	O+
89	8	Male	4+	24	B+
90	84	Male	3+	34	A+

91	6	Female	2+	35	AB+
92	120	Female	2+	28	O+
93	47	Male	4+	28	O+
94	27	Male	4+	27	O+
95	21	Female	4+	32	A+
96	48	Male	4+	28	O+
97	41	Female	2+	30	B+
98	19	Male	2+	37	O+
99	48	Male	4+	38	O+
100	120	Male	4+	34	A+

Note: PVC represents packed cell volume; *patient with missing blood groups were used for the platelet-mediated clumping assay

2.2.2.2 Inclusion and exclusion criteria

A patient was recruited into the study if they were a child less than 12 years of age with a positive microscopy result of ≥ ++ (100-1000 parasites/µL) and whose parent/guardian had given informed consent for their child to participate. A patient was excluded from the study if they had severe anaemia (<15% packed cell volume) in order to not further stress the patient by taking blood. Patients were also excluded from the "Clumping Study" if they had taken anti-malarial medication at least 48 hrs prior to coming to the hospital.

2.2.2.3 ABO blood group typing

ABO blood group antigens were tested by monoclonal ABO blood grouping reagents for Anti-A, Anti-B and Anti-AB (Fortress Diagnostics, product numbers

BGA00010, BGB00010 and BGAB0010). A drop (30-50 µL) of whole blood was mixed with a drop (30-50 µL) of ABO grouping reagent on a grouping microplate. The plate contexts were mixed by tilting at 70˚ angle while gently swirling with care to avoid contamination. Each reaction was observed for at least three minutes for signs of streaming. Anti-A is coloured with acid blue dye, Anti-B coloured with acid yellow dye and Anti-AB is uncoloured. All positive reactions remained as distinct buttons either on the bottom of the well or occasionally sliding down the side.

2.2.3 RNA and DNA extraction from *P. falciparum* infected post-mortem tissues

Three tissues were selected for the purposes of this study: brain, heart and gut. To extract RNA and genomic deoxyribonucleic acid (gDNA), the tissue was weighed and pulverised into a powder with liquid nitrogen using pre-cooled mortar and pestle. Half of the powdered tissue was added to 10X volume Trizol at 1mL of Trizol per 50 – 100 mg tissues for RNA. The remaining half of powdered tissues was placed into 10X volume of lysis buffer for DNA extraction.

RNA preparation

The tissue placed in Trizol was spun at 3,000 rpm for 10 min to pellet insoluble material. The supernatant was transferred into a new tube and incubate at room temperature (RT) for 5 min. 0.2X chloroform was added and the mixture was shaken vigorously for 15 sec, incubated at RT for 3 min, spun at 3,000 rpm for 30-40 min. The

aqueous phase was transferred to fresh tube, mixed gently with isopropanol at 0.5X original Trizol volume (OTV). Equal volumes of the mix was aliquoted into 1.5 mL tubes, incubate at RT for 10 min or left at 4˚C for several days. The tubes were spun at 13,000 rpm for 15 min, aspirated and washed with 1X OTV of 75% ethanol by vortexing then spinning at 12,000 rpm for 5 min. After aspiration and air drying, RNA was resuspended into RNA-resuspension solution (Ambion, product number AM7010), incubated 55 – 60˚C for 10 min, transferred to a new tube and stored -20˚C

DNA preparation

Tissue in the lysis buffer was incubated at 37˚C for 60 min. Proteinase K (20 mg/mL) was added and mixed by swirling at total final concentration of 0.1 mg/mL, then incubated in 50˚C water bath for 3 hrs while mixing occasionally. 1X the total volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added after the mixture was cooled to RT and gently mixed on a wheel for 10 min to emulsify. The phases were separated by spinning at 3,000 rpm for 30 min. The aqueous was slowly transferred to a new tube. 1X the total volume of chloroform was added to the tube, mixed for 30 min and phases separated by spinning at 3,000 rpm for 30 min. The aqueous phase was slowly transferred to a new tube where 0.2X total volume of 10M ammonium acetate was added and mixed. A further 2X total volume of RT ethanol was added and mixed. The precipitated DNA was carefully removed with a sterile loop and transferred to a new tube. All fragment DNA was pelleted by spinning at 3,000 rpm. DNA was washed twice with 1 mL of 70% Ethanol by spinning at 3,000 rpm for 10 min.

After air drying, DNA was resuspend in appropriate volume of 1X Tris Buffer (TE) (10mM Tris, bring to pH 8.0 by HCl and 1mM EDTA), incubated on a rocking platform, 12 – 24 hr to completely dissolve DNA and store at 4˚C

2.2.3.1 DNA-Free RNA preparation

This was done using a DNA free RNA kit (ZYMO research, product number R1013) according to manufacturer instructions. 5 µL 10x DNase I buffer, 2.5 µL RNase-Free DNase I and 22.5 µLl RNase –free water was added to 20 µL of RNA sample was placed in a tube, gently mixed and incubated at 37°C for 10-15 min. RNA was bound to a Zymo-Spin column by centrifuging at 13,000 rpm for 30-60 seconds, followed by two times wash with 200 µL RNA buffer by centrifuging at 13,000 rpm for 30-60 seconds. RNA was eluted using 8-10 µL DNase/RNase-free water and stored at -80°C.

2.2.3.2 cDNA synthesis

Copy DNA (cDNA) was synthesised using a two step protocol from RETROscript reverse transcription kit (Ambion product number AM1710) as described by the manufacturer. 2 µL of random decamers were added to 2 µL RNA together with 8 µL of nuclease-free water to make a final volume of 12 µL. The mixture was heated for 3 min at 75 °C. 2 µL of 10X RT Buffer, 4 µL of deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP), 1 µL of RNase inhibitor and 1 µL of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) were added to the mix on ice to make a final volume

of 20 µL. The mixture was incubated at 42 °C for an hour followed by a 10 min incubation at 92 °C. cDNA was stored at -20 °C .

2.2.3.2.1. cDNA amplicon check using PCR

cDNA amplicon was checked to confirm product formation using a nested PCR assay modified from Duffy *et al*., 2002. The following volumes and concentrations of reagents were added to the nested PCR reaction:

Nest one

2 µL 10X PCR buffer

2.4 µL 25 mM MgCl

1.6 µL 2.5mM dNTPs,

1 µL 20 µM DBLα_dt1for (5'-GGIGCITGYGCICCRTWYMG-3')

1 µL 20 µM DBLα_dt1rev (5'-TCTTCIGYCCATTCCTCGAACCA-3')

0.1 µL *Taq* DNA polymerase (5 U/µL)

1 µL of cDNA template

10.9 µL distilled water

Total reaction volume was 20 µL. PCR was conducted on a GeneAmp thermal cycler 2400 (Perkin-Elmer) or 9700 (Applied Biosystems) using the following conditions;

Initial denaturation: 95°C for 3 min

Denaturation: 94°C for 30 sec

Annealing: 55°C for 30 sec

Extension: 72°C for 1 min

50 cycles of steps 2 – 4

Final extension of 72°C for 7 min

Nest two

2 µL 10X PCR buffer

2.4 µL 25 mM MgCl

1.6 µL 2.5mM dNTPs,

1 µL 20 µM DBLα_dt2for (5'-GCACGMAGTTTYGCIGATATAGG-3')

1 µL 20 µM DBLα_dt2rev (5'-ARATAYTGIGGSACRTARTCIARAT-3')

0.1 µL *Taq* DNA polymerase (5 U/µL)

0.5 µL of nest one PCR product

11.4 µL distilled water

Total reaction volume was 20 µL. PCR was conducted using the same thermocycler used for nest one PCR assays using the following conditions:

1. Initial denaturation: 95°C for 3 min
2. Denaturation: 94°C 30 for sec
3. Annealing: 55°C for 30 sec
4. Extension: 72°C 1 min
5. 50 cycles of steps 2 – 4
6. Final extension: 72°C for 7 min

The formation of a DBL1 DNA fraction of 340-420bp was done by loading 5 µL of the PCR product on a 1% standard agarose gel containing 50 µg/mL ethidium bromide. The resolved bands were visualised under ultraviolet light using a UV-Transilluminator (GelDoc-It Imaging System, UVP, California, USA).

2.2.3.2.2 Whole genome amplification

Whole genome amplification was used to increase low concentrations of parasite DNA. gDNA was amplified using REPLI-g Midi kit (QIAGEN, product number 150023) as described by the manufacturer. 2.5-5 µL of gDNA was placed in a microcentrifuge tube together with Buffer DBL1 and incubated at room temperature for 3 min. Buffer N1 provided with the kit was added followed by 1 µL of DNA polymerase pre-diluted in 29µL reaction buffer on ice. The mixture was incubated at

30°C for 8-16 hours. The REPLI-g polymerase was inactivated by heating the reaction mix at 65°C for 3 min. The gDNA was stored at 4°C for short term use and -20°C for long term storage.

2.3 *P. falciparum* isolates

Two *P. falciparum* laboratory-adapted isolates were used*:* the genome reference isolate 3D7 (MLW parasite bank) (Gardner, Hall et al. 2002) and HB3 (kindly donated by Alister Craig, Liverpool School of Tropical Medicine, UK).

2.3.1 P. falciparum isolation and culturing

2.3.1.1. Preparation of human RBC

Whole blood (type O+) obtained from non-immune volunteers was collected in ethylenediamine tetraacetic acid (EDTA) anti-coagulant at 1.5 mg per 1 mL of whole blood or in a sodium citrate tubes (BD vacutainer, product number 366395), washed 3 times with RT protein-free culture medium (RPMI 1640 (Invitrogen, product number 21875-091) supplemented with 25 mmol/L HEPES and 40 mg/mL gentamycin) by centrifugation for 5-8 min at 1,800 – 2,000 rpm. The RBCs were resuspended in an equal volume protein-free medium to give 50% RBC solution and stored at 4 ˚C to use within 7-10 days.

2.3.1.2. Thawing of glycerolyte-frozen parasites with NaCl

Approximately 1 mL of blood containing 3% ring stage *P. falciparum*-infected erythrocytes (pRBC) was thawed to RT and the pRBC transferred to 50 mL centrifuge tubes with a sterile pipette. 200 µL of 12% NaCl was added slowly, drop-wise, with gentle shaking. After 5 min incubation 5 mL of 1.6% NaCl was added slowly, drop-wise with swirling followed by 5 mL of 0.9% NaCl. The tube was centrifuged a 1,800 rpm at 25 °C for 5 min, washed 2 times with protein-free medium by centrifuging at the same speed before placed in culture.

2.3.1.3. In vitro culture of *Plasmodium falciparum*

For short-term culture, pRBC from patients were pelleted and washed 3 times with 5-10 mL protein-free culture medium by centrifugation at 1,800 rpm for 5 min. The pRBC were placed in a T75 culture flask supplemented with standard malaria culture medium of RPMI 1640 supplemented with 25 mmol/L HEPES, 5% albumax and 40 mg/mL gentamycin to achieve a 5% haematocrit. The flask was incubated for 24 - 36 hrs in a 37 °C incubator. Parasite maturation was examined under a light microscope by a thin blood smear as described below.

Long-term cultures or higher parasitemia were achieved by increasing the volumes of culture medium to 4 times the volume of the packed cell culture.

2.3.1.4 *P. falciparum* thin blood film slide preparation

Thin blood smears are prepared by placing approximately 2 µL of blood on one end of a frosted glass slide resting on a flat surface. The blood was evenly spread across the edge of the second slide by gently touching the drop of blood with the edge of the slide. While holding the second slide at a 45° angle, quickly but gently, without exerting too much pressure on the first slide, slide the blood across the first slide to make a thin film of blood that is evenly spread. Slide was immersed in methanol for 10 seconds, air dried, then stained with 2% Giesma stain for at least 10 min. The excess dye was rinsed with water, air dried and then examined under 90-100x oil emulsion lens on a light microscope

2.3.1.5. Freezing of patient and laboratory isolates with glycerolyte

All steps were carried out at RT unless otherwise specified. Cultures with parasitaemia of ≥5% young ring stages were pelleted by centrifuging at 1,800 rpm for 5 min. 0.33X pellet volume of glycerolyte was added very slowly while gently mixing the tube. After 5 min incubation, 1.33X pellet volume of glycerolyte was added while gently mixing the tube. 1 mL of parasite mix was aliquoted per cryovial, frozen at -80 °C for at least 18 hrs and then transferred to liquid nitrogen tank for long-term storage

2.4 Real-time Polymerase Chain Reactions (RT-qPCR)

2.4.1 Quantification of parasite DNA

RT-qPCR was conducted using a laboratory isolate *P. falciparum* 3D7 gDNA as a control for *var* gene expression as all primers were designed based on the *var* upstream region of this isolate which are found in known proportions, which has been shown to resemble the structure of *var* families in clinical isolates (Lavstsen, Salanti et al. 2003). Parasite DNA was quantified using a 519 bp region of gene PF07_0076, located on chromosome 7 and encoding a protein of unknown function (Daniels, Volkman et al. 2008). Each reaction was carried out in a 10 µL volume using the following volumes and concentrations of reagents:

5 µL 2X SYBR® Green PCR Master Mix

1 µL 2 mM PF07_0076 _for (5'- CGACCCTGATGTTGTTGTTGGA -3')

1 µL 2 mM PF07_0076 _rev (5'-GGCTTTTTTCCATTTCTGTAGTTAAGATTCA -3')

2 µL distilled water

1 µL DNA template

Samples were run on an Applied Biosystems HT7900 real-time PCR System using the following conditions:

1. Denaturation: 95°C for 15 sec
2. Annealing and extension: 58°C for 1 min
3. 50 cycles of steps 1 - 2

Product dissociation curve or melting temperature (T_m) was determined at the following conditions:

1. Denaturation: 95°C for 15 sec
2. Annealing: 60˚C for 15 sec
3. Extension: 95°C for 15 sec

2.4.2. RT-qPCR for *var* group transcription analysis

The procedure for differential *var* group transcriptional analysis was performed by quantitative real-time polymerase chain reaction (RT-qPCR) using primers (Table 2.3) specific for all 3 *var* gene groups and was adapted from (Kaestli, Cockburn et al. 2006). The oligonucleotides were designed according to alignments of 5' untranslated region (UTR) *var* gene sequences from the 3D7 reference isolate and sequences from PNG clinical isolates from Joe Smith (Seattle Biomedical Research Institute, Seattle, WA) and are available at http://www.plasmodb.org (Genbank accession numbers AY462581–AY462851).

Table 2.3. Oligonucleotide primers for amplification of *var* gene regions

***var* gene region (length of amplified product), name**	**Primer sequence (5→3')**
DBL1αrev[a]	CC(A/T)AT(A/G)(G/T)C(A/G/T)GCAAAACT(C/G/T)C(G/T)(A/T)GC
***var* group A (150 bp)**	
upsA1_for[b]	AACTTACCATAAATTATCATCAAA
upsAj_rev	TCACCTACAACAAAT(A/G)TAATAAA
***var* group B (360 bp)**	
17deg_for	CTCAT(A/T)TATAATTTTA(C/G)AAAATA(A/T)A(A/T)AAAAC
RT-17.2_rev	TTA(A/T)GGGAGTAT(A/T)GT(A/G/T)ATATGGTAGAAT
***var* group C (240 bp)**	
RT-5B1.1_for	AATATTCATATTCCCACATT(A/G)TCATATAT
5B1.4_rev	ATTATGTGGTAATATCATGTAATGG

NOTE: [a] Reverse primer used in the primary polymerase chain reaction (PCR).

[b] Forward primers were identical in primary and real-time PCR.

All parasite DNA was standardised to 0.001ng/µl before PCR analysis. Prior to RT-qPCR, 1 µL of DNA was amplified in a primary PCR, to increase sensitivity. DNA was amplified over the *var* 5' UTR–DBL1α in 50 µL volume using the following volumes and concentrations of reagents:

5 µL 10X PCR buffer

3.75 µL 25 mM MgCl

4 µL 2.5mM dNTPs,

1 µL 20 µM Forward primer for upsA, B or C (refer to Table 2.3 for sequence details)

1 µL 20 µM DBL1αrev (refer to Table 2.3 for sequence details)

0.25 µL *Taq* DNA polymerase (5 U/µL)

1 µL of cDNA template

33 µL distilled water

PCR was conducted on a GeneAmp thermal cycler 2400 (Perkin-Elmer) or Veriti thermocycler model 9902 (Applied Biosystems) using the following conditions;

1. Initial denaturation: 94°C for 5 min
2. Denaturation: 95°C for 30 sec
3. Annealing: 52°C for 1 min
4. Extension: 64°C for 70 sec
5. 14 cycles for gDNA and 16 cycles for cDNA of steps 2 – 4

The primary PCR product was checked using 1% standard agarose gel containing 50 µg/mL ethidium bromide as described in section 2.2.1.5 to make sure that it did not exceed the linear range by no visible bands on an electrophoresis gel.

Reactions were done with 2 µL of primary PCR product in 10 µL volume using 5 µL of 2X SYBR® Green PCR Master Mix (Applied Biosystems, product number 4364344), and 900 nmol/L primers per reaction for the respective sequences. RT-qPCR was performed over the *var* group A, B, and C 5' UTR using the Applied Biosystems HT7900 real-time PCR System using the following conditions:

1. Initial denaturation: 94°C for 5 min
2. Denaturation: 95°C for 30 sec
3. Annealing: 54°C for 1 min
4. Extension: 64°C for 70 sec
5. 40 cycles of steps 2 – 4

All runs included a dissociation curve as described in section 2.4.1. All DNA samples were run in triplicate with a cycle-threshold (C_T) within the linear range between 15 and 31 and a melting temperature difference of 1˚C. If all C_T values of *var* group A, B, and C were >31, the sample was discarded and RT-qPCR was repeated. No-template controls (NTC) were amplified in parallel with each reaction plate. If the NTC was positive, the plate was discarded and RT-qPCR repeated. *var* transcript abundance was expressed as proportion of total transcript of all *var* groups. Comparison within groups was done by analysis of variance (ANOVA) and was corrected by the Bonferroni-Dunn method.

2.4.2.1 RT-qPCR Standard curve

Standard curves were linear over a dilution series of 5-6 different dilutions of 3D7 gDNA with concentration ranging between 0.8 - 0.00008 ng/µL, each in triplicate. The PCR efficiency *(E)* was calculated using the formula $E=10(^{1/-slope})-1$. The slope was analysed close to -3.47 as recommended by the manufacturer (Applied Biosystems) to maintain maximum efficiency. The mean efficiencies of 3 independent standard curves with high reproducibility were 100% for *var* group A, 87% for *var* group B, and 96% for *var* group C.

2.4.3 Microarray analysis of cytokine and receptor expression using RT-qPCR

This part of the study was designed to use relative quantitative qPCR to look at genes involved in apoptosis, cytokine production and endothelial receptor expression and investigate any relationship to disease severity (Table 2.4). All primers were validated using 6 fold dilutions of 3D7 gDNA with concentration ranging 1 – 0.00001 ng/µL until all primers amplified at the same melting temperature (T_m) as 3D7 gDNA. Primers had amplification efficiency (E) of between 1.85 and 2 [$E= 10^{(-1/\text{slope of 1 fold dilution of 3D7 gDNA})}$].

All cDNA was adjusted to a concentration of 0.001 ng/µL and reactions were done in 10 µL volume using 5 µL of 2X SYBR® Green PCR Master Mix (Applied Biosystems, product number 4364344) and 900 nmol/L primers per reaction for the

respective sequences. qPCR was performed over the target genes using the Applied Biosystems 7900HT real-time PCR System using the following conditions;

1. Denaturation: 95°C for 15 sec
2. Annealing: 58°C for 1 min
3. Extension: 60°C for 1 min
4. 40 cycles of 1 – 3

Relative quantification was done using the comparative C_T method which determines the change in expression of the target sequence relative to an endogenous control. All DNA samples were run in triplicate using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the endogenous control. Cycle threshold (C_T) for each set of gene-specific primers was compared to those observed for GAPDH. The observed ΔC_T was compared to the expected value from the target gene amplified from 3D7 gDNA.

Table 2.4. Oligonucleotides for target genes of cytokine production and receptor expression.

TARGET GENE	TARGET	FUNCTION
CD36 _for[a]	*Hs* CD36	TTT CCT CTG ACA TT GCA CAA CTC AAG
CD36 _rev[b]	*Hs* CD36	AAA GGC ATT GGC TGG AAG AA
FASLG /CD95L _for	Hs FAS ligand	CAC TTT GGG ATT CTT TCC AT
FASLG /CD95L _rev	Hs FAS ligand	GTG AGT TGA GGA GCT ACA GA
GAPDH _for	*Hs* GAP dehydrogenase	TCA TCT CTG CCC CCT CTG CT
GAPDH _rev	*Hs* GAP dehydrogenase	CGA CGC CTG CTT CAC CAC CT
GP1BB /CD42c _for	*Hs* glycoprotein 1b	AGA ATC TCG ACA CCC TTC TCC
GP1BB /CD42c _rev	*Hs* glycoprotein 1b	ATC TCA CAG TTG CAT AAC CAG G
IFNG _for	*Hs* interferon γ	GCT CTG CAT CGT TTT GGG TTC TCT TG
IFNG _rev	*Hs* interferon γ	CAT TCA TGT CTT CCT TGA TGG TCT CC
ICAM-1 _for	*Hs* ICAM-1	CTG CAG ACA GTG ACC ATC
ICAM-1 _rev	*Hs* ICAM-1	GTC CAG TTT CCC GGA CAA
LICAM _for	*Hs* L1 CAM	GAA CCA TTG ACC TCC GGG
LICAM _rev	*Hs* L1 CAM	CAG CGG TAC TCG CCA TCA T

NCAM /CD56 _for	*Hs* neural CAM	GTC CTG CTC CTG GTG GTT GT
NCAM /CD56 _rev	*Hs* neural CAM	TGA CCG CAA TGC ACA TGA A
PECAM-1 / CD31 _for	*Hs* PECAM-1	GAG CCC AAT CAC GTT TCA GTT T
PECAM-1 / CD31 _rev	*Hs* PECAM-1	TCC TTC CTG CTT CTT GCT AGC T
TFRC1/CD71_for	*Hs* transferring receptor	TGC TGT GAT CGT CTT TTT CTT GA
TFRC1/CD71 _rev	*Hs* transferring receptor	TCA TCC CAA TAT AAG CGA CGT G
TNF _for	*Hs* TNF	CCC AGG GAC CTC TCT CTA ATC A
TNF _rev	*Hs* TNF	GCT ACA GGC TTG TCA CTC GG
TNFR-1/CD120a _for	*Hs* TNF receptor 1	TGC CTA CCC CAG ATT GAG AA
TNFR-1/CD120a _rev	*Hs* TNF receptor 1	ATT TCC CAC AAA CAA TGG AGT AG
TNFR-2/CD120b_for	*Hs* TNF receptor 2	CGC TCT TCC AGT TGG ACT GAT
TNFR-2/CD120b_rev	*Hs* TNF receptor 2	CAC AAG GGC TTC TTT TTC ACC T
VCAM–1 _for	*Hs* vascular CAM	GCT GCT CAG ATT GGA GAC TCA
VCAM-1 _rev	*Hs* vascular CAM	CGC TCA GAG GGC TGT CTA TC
vWF _for	*Hs* von Willebrand factor	AGC CTT GTG AAA CTG AAG CAT
vWF _rev	*Hs* von Willebrand factor	GGC CAT CCC AGT CCA TCT G

2.5 *P. falciparum* and platelet-mediated clumping

2.5.1 Enrichment of parasite mature forms

Parasites were harvested when they had grown to the stage of pigmented trophozoites and the parasitaemia had reached 10-12%. The mature pRBC forms were enriched by plasmagel flotation. Briefly, parasite culture was transferred into a clean sterile 15 mL tube and the pRBC were pelleted by centrifugation at room temperature (RT) at 370 x g for 5 min. The supernatant was carefully removed and the pellet resuspended in a pre-warmed to 37°C solution of gelofusine (BBRAUN, product number FV41513) and RPMI 1640 medium as follows; RPMI making up 20% of total volume, gelofusine making up 30% of total volume and the remaining 50% is the parasite mix suspended in RPMI. The mixture was transferred into a sterile 15 mL tube and allowed to stand for 20 min in a 37°C incubator. The supernatant was transferred to a new sterile 15 mL tube and pelleted by centrifuging at 370 x g for 5 min. Parasitaemia and developmental stage was assessed by thin blood smear and examine under a light microscope as described in section 2.3.2.

2.5.2 Preparation of Platelet-Rich Plasma (PRP)

PRP was prepared from whole blood from a malaria naive host collected in sodium citrate vacutainers. The blood was centrifuged immediately at 250 g for 10 min to pellet erythrocytes and peripheral blood mononuclear cells. The cloudy supernatant containing PRP was carefully transferred in a clean 15 mL tube. The platelets were re-suspended with saline phosphate buffer (PBS) at RT. The platelet were counted with a

Neubauer's haematocytometer and their concentration was adjusted to >300 x 10^3 platelets/µL. PRP was stored at 4 °C for up to a week.

2.5.3 Preparation of Platelet-Poor Plasma (PPP)

PPP was obtained by further centrifuging the supernatant from the PRP preparation from section 2.5.2 at 1500 g for 10 min. PPP was stored at 4 °C for up to a week.

2.5.4 Platelet-mediated clumping assay

The clumping assay was performed with mature pRBC at the trophozoite stage resuspended in a 1.5 mL tube at 5% hematocrit. Acridine orange was added at 20 µg/mL final concentration and PRP at 10% of the total volume. The tube was placed under gentle agitation on rollers at RT. Clump formation was checked by examining 10 µL of pRBC/platelet mix on a slide with a cover slip under fluorescence at 5 min and then every 15 min for a total of 120 min.

2.6 Platelet mobilisation assay

The bottom of the glass petri dishes were soaked overnight in 70% nitric acid in order to provide binding substrate for the platelet-binding substrate 3-aminopropyltriethoxysilane. After rinsing with copious amounts of tap water, the

dishes were dried with anhydrous acetone, immersed twice in 4% solution of 3-aminopropyltriethoxysilane (APES; Sigma), rinsed once with acetone, finally washed four times with distilled water and left overnight at 37°C to dry. PRP using sodium citrate collected blood was prepared as previously described and the platelet count was adjusted to 2 x 10^8 platelets/mL with RT PBS. The bottom of the petri dishes was covered with PRP and incubated for 30 min at room temperature to allow the platelets to settle and spread to form a confluent monolayer. The dishes were washed with room temperature parasite culture medium to remove unbound platelets. The pRBC were enriched for mature forms using gelatin floatation as described in section 2.5.1 and resuspended at 5% haematocrit with RPMI 1640. The pRBC resuspension was evenly added to the platelet-bound plates and co-incubated for an hour at RT. Plates were gently rinsed with PBS to remove unbound pRBC which were also used for RNA extraction. All the bound pRBC were rinsed with RPMI 1640 and RNA was extracted for qRT-PCR as described in section 2.2.1.2.

2.7 Statistical analysis

Statistical analysis was performed using Stata software (Intercooled Stata, version 10). *var* transcript abundance was expressed as the proportion of total transcripts for all three *var* gene groups A, B, and C. Comparison within groups was done by analysis of variance (ANOVA) and was corrected by the Bonferroni-Dunn method. Associations between *var* group proportions and clinical outcome were analyzed using the Kruskal-Wallis test or the Fisher's exact test.

2.8 Chemicals and reagents

Table 2.5. List of chemicals and reagents

Item	Product number	Manufacturer/Supplier
0.2 mL cap strips	732-3590	VWR
0.2 mL tube strips	732-3588	VWR
10 uL filter tips	F104-96RS-10	Porex, WhiteSci
10 uL filter tips	F104-96RS-10	Porex, WhiteSci
1000 uL filter tips	F119-NXL-R100S-1000	Porex, WhiteSci
100bp DNA ladder	15628-050	Invitrogen
20 uL filter tips	F114-R100S-20	Porex, WhiteSci
200 uL filter tips	F108-96RS-200	Porex, WhiteSci
2-Propanol, 99%, Molecular Biology grade	I9516-500ML	Sigma
3-aminopropyltriethoxysilane	15108500	Acros Organics
Acridine orange stain	P/L780/05	Fisher
Adhesive PCR foil	732-4838	VWR
Agarose, low melting	BPE1360-100	Fisher
Albumax II	11021-037	Invitrogen
Anhydrous acetone, Molecular Biology grade	A-0560-08	Fisher
Butterfly needles, 21 g x 10 cm	FSB458	NHS
Cannula (IV) blue 22g x 25mm Jelco (4030)	FSN017	NHS
Centrifuge tubes , 50mL Falcon	358206	BD
Centrifuge tubes, 15 mL	FB55950	Fisher
Centrifuge tubes, 15ml Falcon	352097	BD
Centrifuge tubes, 50 mL	FB55957	Fisher
Culture flask, 175 cm2	TKT-130-215S	Fisher

Culture flask, 25 cm2	TKT-130-150L	Fisher
Culture flask, 25 cm2, filtered	TKT-130-170F	Fisher
Culture flask, 80 cm2	TKT-130-190W	Fisher
Culture flask, 80 cm2, filtered	TKT-130-210T	Fisher
DNA-free RNA kit	R1013	Cambridge Bioscience
dNTPs set	U1240	Promega
Ethanol, absolute, Molecular Biology grade	E7023	Sigma
Ethidium Bromide, stain	2149846	Sigma
Gentamicin, 50 mg/ml	15750-037	Invitrogen
Giemsa stain, modified	GS500-500ML	Sigma
Glass petri dishes	PDS-100-011U	Anumbra
HEPES Buffer Solution 1M, liquid	15630-056	Invitrogen
L-glutamine	G7513	Sigma
Lymphoprep	NYC-1114545	Axis-Shield
MicroAmp optical 384-well reaction plates	4309849	Applied Biosystems
MicroAmp optical 96-well reaction plates	4306737	Applied Biosystems
Needles, disposable, 21 gauge	SZR-175-530R	Fisher
Nitric acid	N1680CCo2500	Associated Chemical Ent.
Nuclease-free water	P1193	promega
Pipettes, 1 mL	PN1E1	ALP, WhiteSci
Pipettes, 10 mL	FB55484	Fisher
Pipettes, 2 mL	PN2E1	ALP, WhiteSci
Pipettes, 5 mL	FB51889	Fisher
REPLI-g Mini kit	150023	Qiagen
RETROscript cDNA synthesis kit	AM1710	Ambion
RPMI 1640 medium (1x) with L-glutamine	21875-091	Invitrogen

Sodium citrate tubes	366395	BD
SYBR® Green PCR Master Mix	4364344	Applied Biosystems
Taq Polymerase kit	201207	Qiagen
TaqMan	4364338	Applied Biosystems
Trizol	15596-026	Invitrogen
Trypan blue	T8154	Sigma
Tubes, 1.5mL	FB74031	Fisher
Tubes, culture, 12 x 75 mm, PP	TKV-182-035N	Fisher
Tubes, culture, 17 x 100 mm, PP	TKV-182-081G	Fisher

Chapter 3

3. DIFFERENTIAL EXPRESSION OF *VAR* GENE GROUPS IN *P. FALCIPARUM* INFECTIONS IN MALAWIAN PEDIATRIC MALARIA PATIENTS

3.1 INTRODUCTION

P. falciparum can affect the host in different ways. The severity of the infection varies depending on host as well as parasitic factors. Infection can be asymptomatic or manifest via a range of signs and symptoms, including severe anaemia and cerebral dysfunction which can both be lethal to the patient. One of the factors contributing to the severity of pathology of *P. falciparum* is the ability of mature parasite forms to cytoadhere to various host endothelial receptors in deep vascular beds leading to microvessel obstruction and damage in various organs. This process is thought to be a host immunity avoidance strategy, allowing the parasite to circumvent splenic clearance (Smith, Chitnis et al. 1995; Kyes, Horrocks et al. 2001; Kaestli, Cortes et al. 2004).

Cytoadherence is mainly mediated by PfEMP1), a family of antigens with assemblies of semi-conserved DBL and CIDR adhesive domains. The ~60 encoding *var* genes are not all expressed simultaneously but instead one at a time in a mutually exclusive manner (discussed in Chapter 1, section 3.3.3). This allows the parasite to switch between surface expression of the various *var* gene products at rates as slow as 0.03% per generation or fast as 18% per generation, resulting in antigenic variation, while maintaining or changing the adhesive properties. It is through this clonal

antigenic variation that the parasite is capable of evading the host immunity (Peters, Fowler et al. 2002; Kaestli, Cortes et al. 2004).

As previously discussed in Chapter 1, in the *P. falciparum* reference genome 3D7, *var* genes can be classified into three main groups: A, B and C according to their 5' upstream and intron sequences (Voss, Thompson et al. 2000; Gardner, Hall et al. 2002). The majority of *var* genes (76%) belong to group B genes located subtelomerically, followed by the *var* group C (15%) arranged in clusters on chromosomes 4, 6, 7 and 8. The least numerous of the *var* genes (10%) belong to the more structurally diverse group A, which are mostly located subtelomerically, on most chromosomes, and have a distinct direction of transcription towards the telomeres (Voss, Thompson et al. 2000; Gardner, Hall et al. 2002; Lavstsen, Salanti et al. 2003).

In the past, various studies using clinical isolates have tried to investigate the association between disease outcome and binding affinity for host endothelial receptors (Turner, Morrison et al. 1994; Fried and Duffy 1996; Newbold, Warn et al. 1997). Bull et al (2000), using agglutination assays, showed that parasites isolated from children with severe malaria had higher agglutination frequencies compared to isolates from children with mild malaria and the agglutination frequency decreased with age (Bull, Kortok et al. 2000). In 2005, the same group was able to class *var* genes from Kenyan field isolates and laboratory isolates into biologically meaningful subsets based on cysteine-containing small blocks of semi-conserved sequences, thus providing some evidence of the existence of *var* gene semi-structuring (Bull, Berriman et al.

2005). It was from these results that the question of the preferential involvement of *var* groups A, B and C in various clinical syndromes was raised.

Table 3.1. Summary of outcome of clinical *var* gene group studies

Author/year/locale	*var* group A	*var* group B	*var* group C
Bull *et al*/ 2005/Kenya	no difference		
Kaestli *et al*/2006/PNG	rosettes	clinical malaria (severe & mild)	asymptomatic
Kyriacou *et al* /2006/Mali	cerebral malaria	hyperparasitaemia	
Rottmann *et al* /2006/Tanzania	severe malaria	clinical malaria (severe & mild)	
Warimwe *et al*/2009/Kenya	severe malaria		
Kalmbach *et al*/2010/Gabon		cerebral malaria	
Avril *et al*/2012/Kenya	cerebral malaria		
Lavstsen *et al*/2012/Tanzania	severe malaria		

Two studies, one in Papua New Guinea (PNG) and the other in Tanzania, comparing asymptomatic infections to clinical malaria (mild, severe), found a preferential expression of *var* gene groups A or B in clinical cases (Kaestli, Cockburn et al. 2006; Rottmann, Lavstsen et al. 2006). Kaestli *et al.* (2006) also found v*ar* group A to be associated with rosetting isolates (summarised in Table 3.1). Another study conducted in Kenya found no difference in *var* group expression between severe and mild disease (Bull, Berriman et al. 2005), and in Mali an association between *var* gene group and cerebral malaria was found in children (Kyriacou, Stone et al. 2006). *var* gene

group B was found to be associated with cerebral malaria in Gabon (Kalmbach, Rottmann et al. 2010) and both severe and uncomplicated malaria in PNG (Kaestli, Cockburn et al. 2006) and Tanzania (Rottmann, Lavstsen et al. 2006). *var* gene group B has also been associated with hyperparasitaemia in Mali (Kaestli, Cockburn et al. 2006). *var* gene group C has mainly been associated with asymptomatic malaria (Kaestli, Cockburn et al. 2006) but was found to be associated with hyperparasitaemia in Mali (Kyriacou, Stone et al. 2006) and cerebral malaria in Gabon (Kalmbach, Rottmann et al. 2010). Interestingly, after classifying *var* genes from Kenyan field and laboratory isolates into biologically meaningful subsets based on small blocks of semi-conserved cysteine sequences, no association was found between the *var* groups and the various clinical presentations. However, an association was found between the presence of only 2 conserved cysteines in the DBLα domain and the rosetting phenotype (Bull, Pain et al. 2005).

Although these studies have provided evidence that these *var* subtypes are associated with various disease outcomes, the results have been contradictory. No definite associations can be drawn. Several factors may contribute to these differences in findings: 1) all of the studies used peripheral blood samples which may not give an accurate representation of the sequestered parasite populations, 2) geographic (and potential endemnicity) differences resulting in *var* genes with different disease causing abilities 3) the use of different *var* gene group classifications and tools to study them, such as primer sets, and 4) differing definition of malaria disease states (Nogueira, Wunderlich et al. 2001; Fowler, Peters et al. 2002; Bull, Berriman et al. 2005; Albrecht,

Merino et al. 2006; Fowler, Chavchich et al. 2006; Barry, Leliwa-Sytek et al. 2007). The most consistent outcome has been identifying the association of *var* gene group A with severe forms of malaria (Table 3.1) which through recent findings supports the idea that specific subset of group A *var* genes mediates parasite adherence to human brain endothelia (Avril, Tripathi et al. 2012; Claessens, Adams et al. 2012; Lavstsen, Turner et al. 2012). Thus, CD36 binding is not a major factor and ICAM-1, although important in cerebral binding, is not necessary for widespread binding of pRBC to brain endothelia (Avril, Tripathi et al. 2012).

All *var* gene studies point to the general important fact that *P. falciparum* field isolates are highly dynamic and variable in their *var* transcription, supporting the observation that certain *var* gene repertoires are able to recombine during the sexual phase of the life cycle in the mosquito (Su, Ferdig et al. 1999) and during both meiosis and mitosis, creating diversity even among closely related genomes (Ward, Clottey et al. 1999; Freitas-Junior, Bottius et al. 2000; Taylor, Kyes et al. 2000).

We have been conducting a clinicopathological study of fatal paediatric malaria in Blantyre, Malawi, since 1996 (Taylor, Fu et al. 2004). In a previous study, we have shown that pRBC in the organs tend to be more genetically complex than in peripheral blood sample, which is a snapshot in time, and contains a subset of all parasite populations found in host. This study also demonstrated that, when compared to children with non-malarial causes of death, fatal CM patients have less complex infections, with genetically homologous parasites present in multiple organs (Montgomery, Milner Jr. et al. 2006). We have also shown that there is dominant

expression of particular set of *var* genes within a tissue; these dominant expression patterns vary between organs. However, some of the dominant *var* gene patterns are detected in the same organs of other patients from the same malaria season (Montgomery, Mphande et al. 2007). This chapter describes the investigation of differential *var* gene group expression of *P. falciparum* in the organs and the relation of those expression patterns to clinical disease presentation.

OBJECTIVES

The objectives of this study are to:

i. Determine the *var* group dominantly expressed by parasites that are found sequestered in the brain, heart and gut
ii. Determine the dominant *var* group expressed in sequestered parasites isolated from children with different clinical presentations of cerebral malaria
iii. Compare *var* transcripts in sequestered and circulating parasites

3.2 MATERIALS AND METHODS

3.2.1 Study design, materials and methods

A full description of all the methods used in this study has already been outlined in Chapter 2

3.3 RESULTS

3.3.1 Parasite *var* gene distribution

In total, 20 patients were selected for *var* gene group expression analysis. Patients were chosen based on diagnostic classification (Taylor, Fu et al. 2004) and from data extracted from the pilot study on *var* expression (Montgomery, Mphande et al. 2007). This had raised the suggestion of limited diversity of *var* genes within a malaria season, and so the patients were chosen at a fixed ratio over five malaria seasons. The ratio of 2 CM2 : 1 CM1 : 1 PC was based on the observation that CM1 cases are found at half the rate of CM2 cases (Taylor, Fu et al. 2004). One PC case was later revised as CM2 based on histological examination, leaving us with a final selection of CM1 (n=5), CM2 (n=11) or PC (n=4).

All patients were recruited from four different malaria seasons between 1999 and 2004 to allow comparison of parasite genetic make-up with seasonality (Table 3.2). There were no significant difference in age, time to death or parasitaemia, either at admission or death, between the three diagnostic groups (age and diagnostic group, p=0.37; time to death and diagnostic group, p=0.32; admission parasitaemia and diagnostic group, p=0.24; final parasitaemia and diagnostic group, p=0.95; Kruskal-Wallis test).

3.3.2 Restricted genetic diversity of CM patients

For each of the patients, *var* gene group expression was analysed in three tissues: brain, heart and gut. From our previous study (Montgomery, Milner Jr. et al. 2006), using *msp* genotyping, we found no evidence of restricted *P. falciparum* genetic diversity in the brain or particular genetic variants specifically sequestered in this organ. This work is focused on determining whether parasites sequestering in the brain express a particular *var* gene group, which might be associated with CM. The heart is a reasonable comparative tissue as it displays less diversity of *var* transcripts than the brain (Montgomery, Mphande et al. 2007). The gut was examined because it is another organ of high parasite density (Montgomery, Milner Jr. et al. 2006).

Table 3.2. Clinical details of patients in main study

Diagnosis	Case no.	Year of admission	Age[a]	HIV status	Time to death[b]	Admission parasitaemia[c]	Final parasitaemia[c]
CEREBRAL MALARIA							
CM2	28	1999	61	-	00:30	424,000	424,000
	34	1999	70	-	02:45	74,319	74,319
	61	2002	26	-	04:15	42,027	42,027
	62	2002	10	-	01:10	1,056,607	1,056,607
	63	2002	79	-	16:10	8,212	400
	64	2002	60	+	07:00	28,842	28,842
	68	2003	156	+	15:25	28,080	4,680
	75	2003	144	+	07:50	215,300	36,250
	78	2003	15	-	02:00	637,000	637,000
	82	2004	31	-	34:00	197,820	0
	83	2004	26	-	13:45	22,000	6,000
mean			*61.6*		*09:32*	*247,837*	*210,011*
st dev			*49.4*		*09:56*	*334,794*	*350,186*
CM1	37	1999	6	+	38:00	616,400	572,880
	38	1999	84	-	35:40	782,320	5,474
	74	2003	103	+	05:25	280,000	280,000
	79	2003	79	+	04:40	34,400	34,400
	84	2004	106	+	21:07	201,829	834
mean			*75.6*		*20:58*	*382,990*	*66,142*
st dev			*40.6*		*16:55*	*307,769*	*120,250*
PARASITAEMIC CONTROLS							
[d]	31	1999	39	+	22:00	159,434	188,432
[d]	45	2000	28	-	16:10	100,519	197
	77	2003	7	-	04:05	66,844	66,844
[e]	80	2004	63	-	12:00	23,700	23,700
mean			*34.3*		*13:34*	*87,624*	*79,668*
st dev			*23.3*		*07:32*	*57,274*	*78,700*

[a] months, [b] hours:minutes, [c] parasites/µL in peripheral blood, [d] pneumonia (*Streptococcus*), [e] meningoencephalitis

Two different techniques were used to genetically characterise the infecting isolates: *msp* typing (provides the MOI without quantitatively identifying the VSA (Snounou, Zhu et al. 1999)) and a SNP assay called barcoding (detects the major 90% of genetic variants and classifies the infection as homo- or heterozygous (Daniels, Volkman et al. 2008)) on 16 of the 20 hosts. In accordance with other studies in Malawi (Dembo, Phiri et al. 2006; Bruce, Macheso et al. 2008), we found a mean of 2.3 ±0.7 genetic variants per patient, and 1.6-1.8 ±0.9 variants per tissue, with no significant differences in MOI between tissues, diagnostic groups or season. CM infections commonly consisted of a single genetic variant that was the major isolate present in all three tissues in agreement with previous studies. Only 1/5 (20%) in CM1 and 3/8 (37.5%) CM2 patients had evidently mixed infections. In contrast, 2/3 (67%) PC cases examined by *msp* genotyping contained more than one genetic variant and all three patients had major and minor alleles detected at at least one loci (Figure 3.1).

In order to ensure that the genomic composition of the *var* subgroups was similar to the 3D7 reference genome and other sequenced genomes and to exclude primer bias, the ratio of *var* gene groups A, B and C in the gDNA of all the 20 malaria cases were quantified by qRT-PCR. The genomic distribution of the three *var* subgroups was similar among hosts from different clinical diagnostic groups as well as between the brain, heart and gut tissues, with 7% of the overall *var* genes amplified belonging to *var* group A, 76% to *var* group B and 16% to *var* group C, as shown in the brain (Figure 3.2A). This was in agreement with data from other sites and studies (Figure 3.2B) (Kaestli, Cockburn et al. 2006).

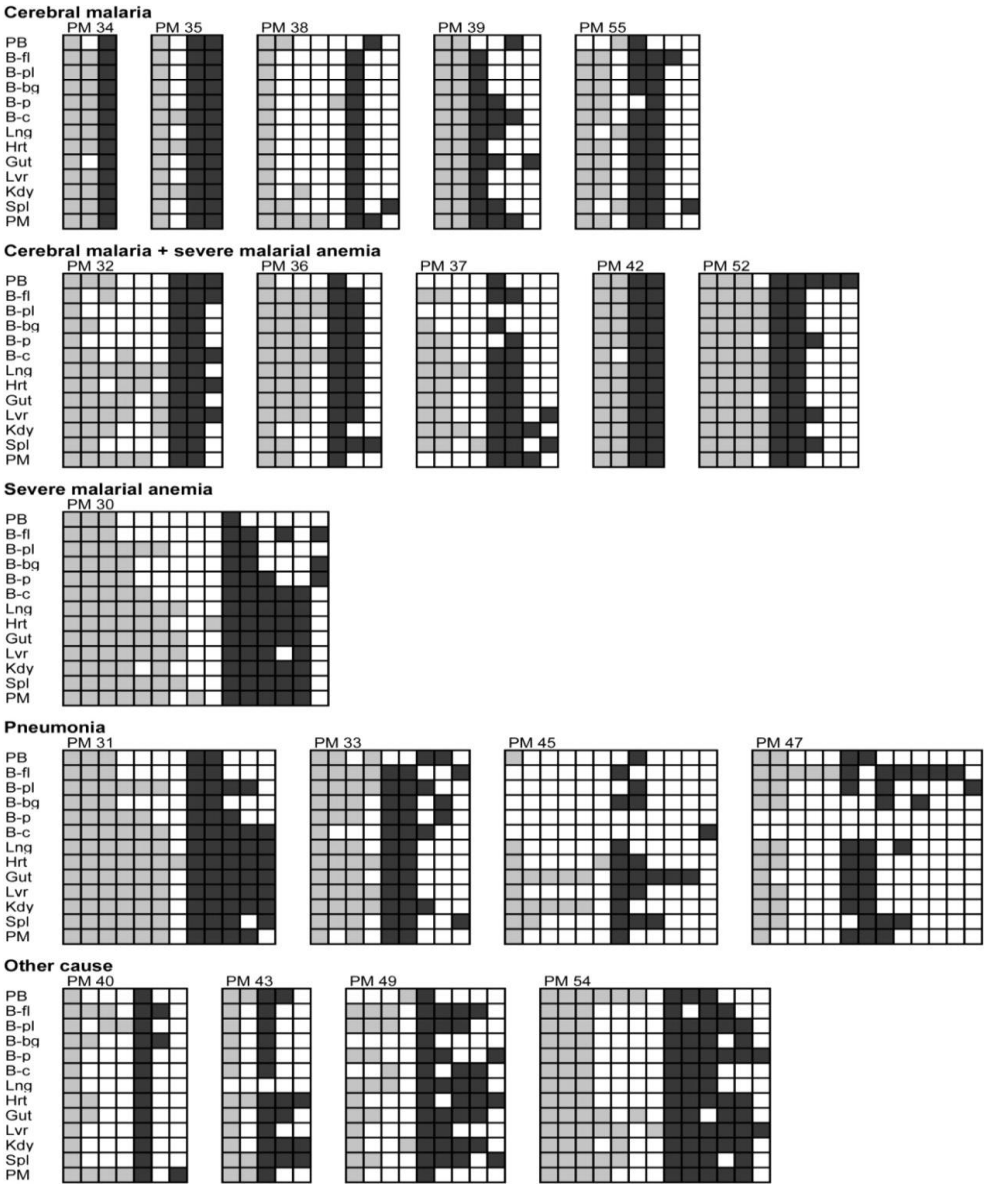


Figure 3.1. Distribution of *P. falciparum* merozoite surface protein *(msp)* 1 and 2 genotypes in the tissues of 19 parasitaemic children. Each chart has the patient number above and tissue type on left and represents data from a single patient. A genotype detected by PCR is represented by each shaded square. Vertical alignment represents genotypes of the same electrophoretic mobility within a chart, but not between charts. Light gray shading denotes *msp*1 alleles, and dark gray shading denotes *msp*2 alleles. B-bg, brain basal ganglia (caudate); B-c, cerebellum (tonsils); B-fl, brain frontal lobe; B-p, pons (brain stem); B-pl, brain parietal lobe; Gut, jejunum and right colon of gut; Hrt, left ventricle of heart; Kdy, right kidney; Lng, right upper lung; Lvr, liver; PB, peripheral blood; PM, pectoralis muscle; Spl, spleen (*From Montgomery et al. J Infect Dis (2006) 194 (1): 115-122)*

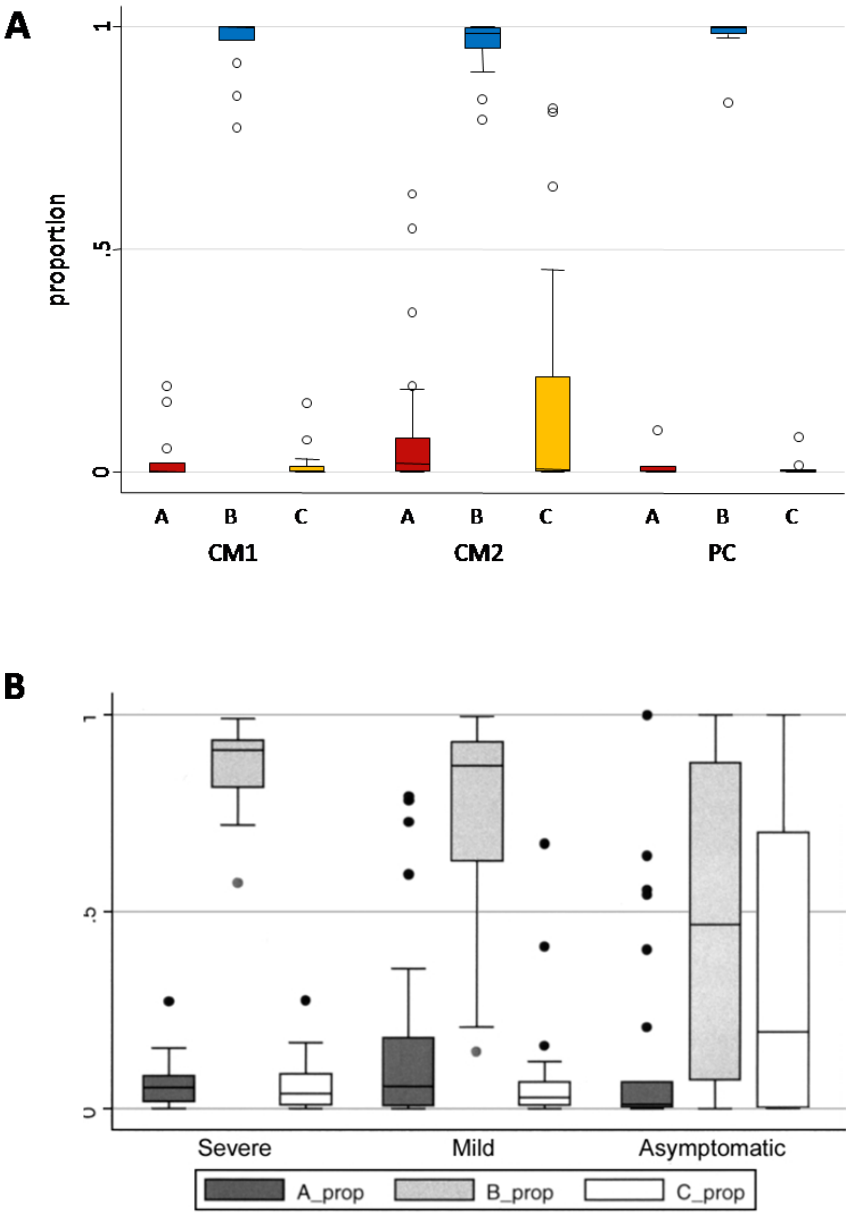


Figure 3.2. Genomic distribution of *var* gene groups in parasites from children with different malaria diagnoses. (**A**) genomic *var* gene distribution in the brains of CM1 (n=5), CM2 (n=11) and PC (n=4) patients (**B**) genomic *var* gene distribution in different malaria diagnostic groups determined by Kaestli *et al.* 2006. Both graphs display *var* gene group B as the majority in the tissue and the diagnostic group. The box plots outline 25th and 75th percentiles, with the median indicated as a line inside each box and the 5th and the 95th percentiles are illustrated by the whiskers. Outliers are indicated by the open circles.

3.3.3 Comparison of *var* transcripts between tissues and diagnostic groups

After verification that the distribution of the three main *var* groups was similar to 3D7, qRT-PCR was used to determine the levels of expression of the main *var* subgroups in parasites found sequestered in the tissues of the same 20 patients. Highly significant differences were observed in the expression levels of the 3 *var* subgroups in parasites from children with classical cerebral malaria (CM2), when compared with those found in children with cerebral malaria with sequestered parasites only (CM1).

In CM2, group A *var* genes were expressed at high levels in the brain compared to group B and C genes (p=0.0001, ANOVA Bonferroni; Figure 3.3A), consistent with some peripheral blood studies linking group A expression with severe forms of disease (Kyriacou, Stone et al. 2006; Rottmann, Lavstsen et al. 2006). Group A genes were also highly expressed in the heart and gut. The second most highly expressed were the group C *vars* with the group B *vars* being the least expressed (p=0.0001, ANOVA Bonferron; Figure 3.3A). A similar pattern of *var* expression was observed in parasitaemic controls (PC) cases (Figure 3.3B).

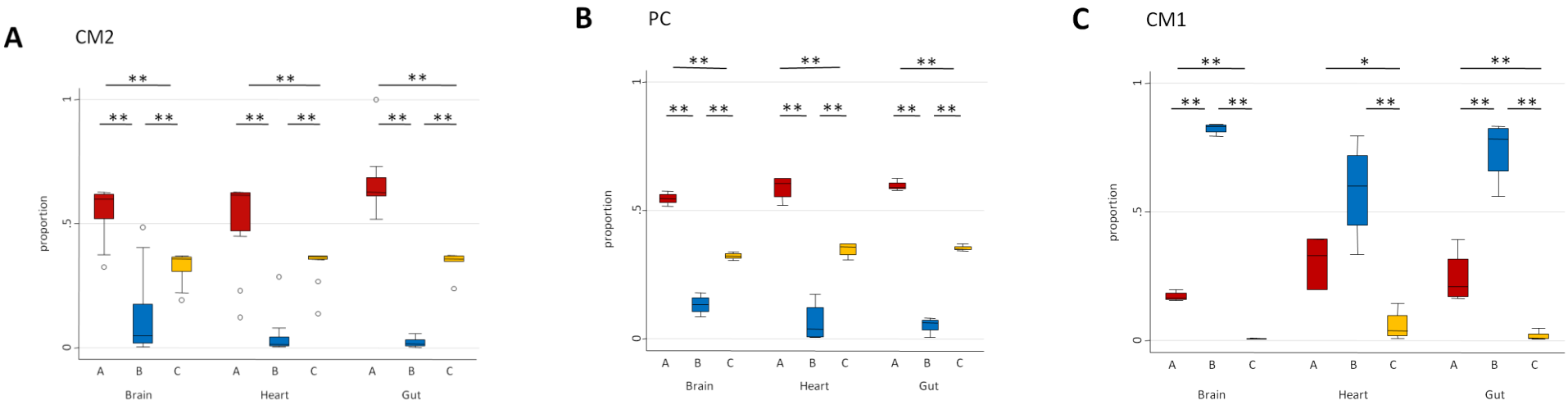


Figure 3.3. Transcript abundances of *var* gene groups in parasites found in the tissues of children in different diagnostic groups. Transcripts are shown as proportions of the total number of *var* gene transcription for parasites found in each tissue of the patients. Box plots of the proportion of *var* gene groups A, B and C in the brain, heart and gut of CM2 (n=11 for all tissues) (A), CM1 (n=5 for brain and gut, n=4 for heart) (B) and PC (n= 4 for all tissues) (C) patients. The box plots outline 25th and 75th percentiles, with the median indicated as a line inside each box and the 5th and the 95th percentiles are illustrated by the whiskers. Outliers are indicated by the open circles. (ANOVA; $**p \leq 0.001$, $*p \leq 0.05$ after Bonferroni correction)

The pattern of expression in CM1 was different from CM2 and PC, with group B *var* being highly expressed (p=0.0001, ANOVA Bonferroni) and group C being the least expressed regardless of tissue (Figure 3.3C). The difference in *var* expression between the CM1 and CM2 is not due to parasite density as there were no significant differences in parasite densities between these two diagnostic groups at admission or at the time of death (p=0.23 and p=0.77, respectively, ANOVA Bonferroni). Peripheral parasitaemia is not a good indicator of sequestered pRBC density. However, Taylor *et al* have shown that both CM1 and CM2 have similar cerebral pRBC density compared to PC (Taylor, Fu et al. 2004). The parasitaemia between CM2 and PC was similar at admission and at death (p=0.80 and p=0.7, respectively, ANOVA Bonferroni) despite the outstanding differences in pathology. These results overall suggest that the distribution of *var* transcription is irrespective of the number of pRBC present or the severity of symptoms.

3.3.4 Influence of HIV on *var* gene expression

The results of the initial *var* gene group expression analysis of the 20 patients were intriguing, considering there were no significant differences in the clinical admission details of patients. CM1 accounts for approximately 25% of patients clinically diagnosed with cerebral malaria, and currently can be distinguished from CM2 only by post-mortem examination. Both CM1 and CM2 can be distinguished from PC by post-mortem examination or malaria retinopathy, one of the entities of the ocular fundus

differences displayed in severe falciparum infections (reviewed in (Lewallen, Harding et al. 1999)) that can precisely distinguish the different subsets of cerebral malaria (Taylor, Fu et al. 2004). 80% (n=4) in Table 3.3 and all patients in Table 3.4 (n=2) of the CM1 patients had a positive HIV status, a potential contributing factor. This is characteristic of clinically diagnosed CM patients in the parent study in which 19% of CM2 and 6% of PC hosts were HIV+, whereas nearly two thirds (62%) of CM1 cases were infected (unpublished observations).

In order to determine whether the presence of HIV in the patient influenced *var* expression, both data sets in Tables 3.3 were split according to HIV status. Eight out of the 20 patients (40%) were HIV positive and most belonged to the CM1 group (80%). Comparing age, time to death and parasitaemia, both at admission and at death, between HIV positive and HIV negative patients, there were no significant differences. (Age and HIV status, p=0.0521; time to death and HIV status, p=0.1863; admission parasitaemia and HIV status, p=0.8688; final parasitaemia and HIV status, p=0.3637; Kruskal-Wallis test).

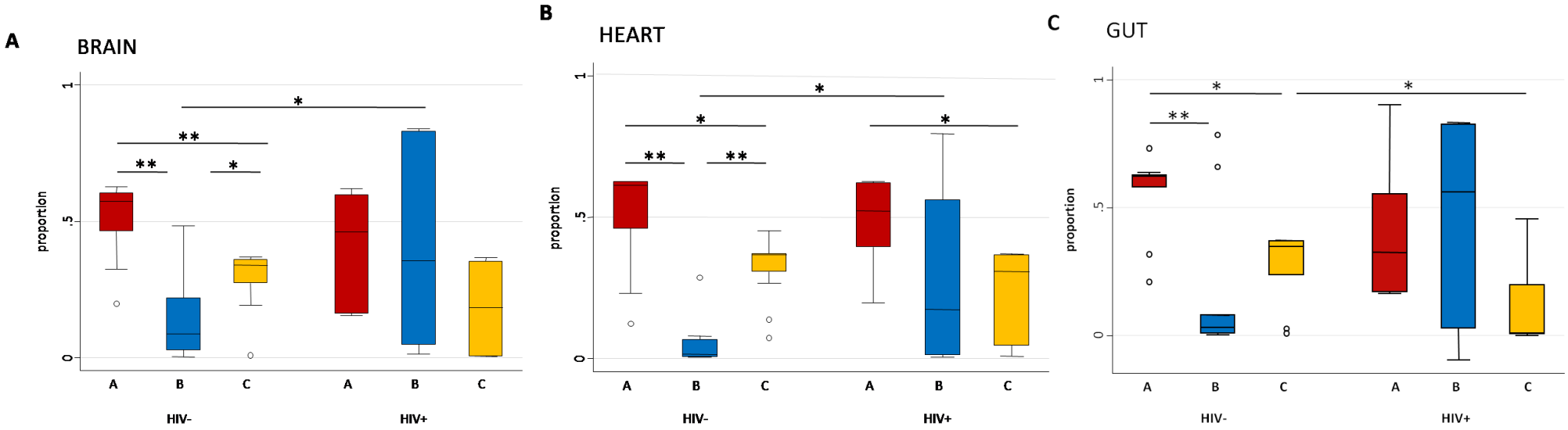


Figure 3.4. Transcript abundance of *var* gene groups in parasites in the tissues in HIV positive and HIV negative cerebral malaria patients. Transcripts are shown as proportions of the total number of *var* gene group transcripts for parasites found in each tissue of each patient. Proportion of *var* gene groups A, B and C transcripts in the brain (A), heart (B) and gut (C). The box plots outline 25th and 75th percentiles, with the median indicated as a line inside each box and the 5th and the 95th percentiles are illustrated by the whiskers. Outliers are indicated by the open circles. (ANOVA; $^{**}P \leq 0.001$, $^{*}P \leq 0.05$ after Bonferroni correction)

The antigen expression pattern in HIV- cases closely resembled that of CM2 and PC in section 3.4.2 in all the tissues, with group A highly expressed and group B genes the least expressed (group A vs B, p=0.0001; group A vs C, p=0.001; ANOVA Bonferroni) (Figure 3.4). There was substantial variation in the HIV+ data, with no major differences between *var* group expression in the brain and heart of HIV+ individuals. However, in the gut of HIV+ hosts, similar patterns of *var* expression were observed as in HIV- hosts, with the group A *vars* being significantly higher expressed than group B (p=0.0001) and C (p=0.001).

Comparing HIV+ versus HIV-, there were no differences in group A and C expression in all the tissues. However, significant differences were observed in group B, with expression being highest in HIV+ especially in the brain (p=0.046, ANOVA Bonferroni) and heart (p=0.034, ANOVA Bonferroni) as was seen in CM1. Group A was the least expressed and similar in the HIV+ and HIV- children. While expression of both B and C *var* gene groups were elevated, only *var* gene group B transcripts were significantly higher than *var* gene group C in the HIV+ (p=0.020, ANOVA Bonferroni).

Unfortunately we do not have any measures of HIV disease severity in these patients, and so this data presumably spans a range of immunological deficiency. However, the significant differences in *var* expression in HIV negative patients suggest that HIV infection indirectly impacts pRBC *var* gene expression, perhaps through eliciting changes in the immune and inflammatory responses that upregulates the expression of certain receptors on the endothelial cells. This in turn could influence the sequestration of certain parasites.

These results lead us to speculate that in the absence of HIV, CM patients are likely to develop into classical cerebral malaria as seen in CM2. The odds ratio indicated a marginal significance, with CM1 patients 12 times more likely to be HIV positive than CM2 (OR: 12.0 with 95% CI (1.0-136.7) *p*=0.05728 (Table 3.3)). Nevertheless, based on the small sample size, the study may not be powered to provide enough evidence. More information is also required on the severity of the HIV symptoms in both CM1 and CM2 groups in terms of their CD4+ cells and viral load, as this could help explain the high parasitaemia and pathology, as has been previously shown (Migot, Ouedraogo et al. 1996; Mermin, Lule et al. 2004).

Table 3.3. Patient HIV status in the CM1 and CM2 clinical diagnostic groups

HIV status	CM1	CM2	Total
positive	6	4	10
negative	1	8	9
Total	7	12	19

Note: The odds ratio (OR) calculated from the total number of children that were HIV positive and total number of children that were HIV negative. Children in CM1 are more likely be HIV positive OR: 12.0 with 95% CI (1.0-136.7) *p*=0.0573 and less likely to the HIV negative OR: 0.0833 with 95% CI (0.0073-0.9500) compared to CM2

3.4 Comparison of *var* transcripts: tissues versus peripheral blood:

What is wrong with this picture?

Unfortunately, there was no peripheral blood (PB) obtained from the 20 patients to allow comparison of antigen expression between the tissues and PB due to post-mortem collection procedures of the parent study at this time. We therefore recruited 5 additional patients whose PB and tissue biopsies were both available for this analysis. The 5 patients were recruited between the 2006-2008 malaria seasons (Table 3.4) following the selection criteria described in section 3.3.1. We found no significant differences in age, time to death and parasitaemia, both at admission and death, between the three diagnostic groups (age and diagnostic group, p=0.8187; time to death and diagnostic group, p=0.2231; admission parasitaemia and diagnostic group, p=0.8187; final parasitaemia and diagnostic group, p=0.2231; Kruskal-Wallis test).

Table 3.4 Patients in additional study with matched peripheral blood

Diagnosis	Case no.	Year of admission	Age[a]	HIV status	Time to death[b]	Time to autopsy[b]	Admission parasitaemia[c]	Final parasitaemia[c]
CM2	96	2007	31	-	10:55	11:55	236	236
	98	2008	96	+	32:40	10:40	471,056	17,610
CM1	95	2007	11	+	04:00	08:20	688,867	688,867
	99	2008	89	+	02:25	04:05	78,904	78,904
PC	92	2006	39	-	30:40	11:50	90,960	90,960

[a] months, [b] hours:minutes, [c] parasites/μL in peripheral blood

As previously described, the relative proportion of genes in the three main *var* groups was measured in the genomic DNA to check similarity to the 3D7 genome. The *var* gene distribution was different from what was observed for the 20 patients; with no significant differences in the *var* gene group distribution between CM1 and CM2 (Figure 3.5).

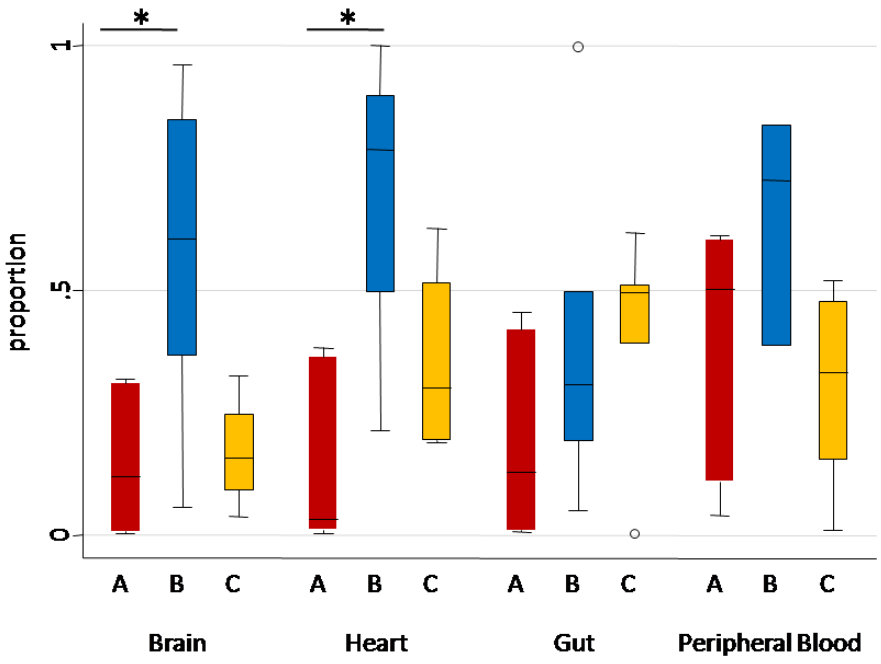


Figure 3.5. Genomic distribution of *var* gene groups amplifications of parasites isolated from the brain, heart, gut and peripheral blood of children with different malaria diagnoses. The box plots outline 25th and 75th percentiles, with the median indicated as a line inside each box and the 5th and the 95th percentiles are illustrated by the whiskers. Outliers are indicated by the open circles. (ANOVA; *$p \leq 0.05$ after Bonferroni correction).

Only PC showed a significantly high distribution of group B (p=0.0001, ANOVA Bonferroni) compared to the group A and B. However, the number of group B was much more pronounced in the tissues, especially the brain (A vs B, p=.041) and heart (A vs B p=0.028, ANOVA Bonferroni) while Group C numbers were slightly raised in CM1 compared to PC (CM1 vs PC, p=0.024, ANOVA Bonferroni) and in the gut although this difference was not statistically significant.

Comparison of *var* expression between the tissues and peripheral blood showed a different expression pattern as in the diagnostic groups described above. There were no significant differences in *var* expression within the tissues but Group A *vars* were highly expressed in PB compared to the tissues (p=0.011, ANOVA Bonferroni) (Figure 3.6). In our previous study (Montgomery, Milner Jr. et al. 2006) using *msp* genotyping, we have shown that some circulating parasites are also detected in the different organs, implying that the genotype of the parasites is common to tissues and peripheral blood. Some *var* gene studies using peripheral blood have found an abundance of both groups A and B in children with mild and severe malaria (Kaestli, Cockburn et al. 2006; Rottmann, Lavstsen et al. 2006) and the expression of *var* gene group C can be similar between different clinical malaria diagnostic groups (Rottmann, Lavstsen et al. 2006) as is the case in this *var* gene expression analysis.

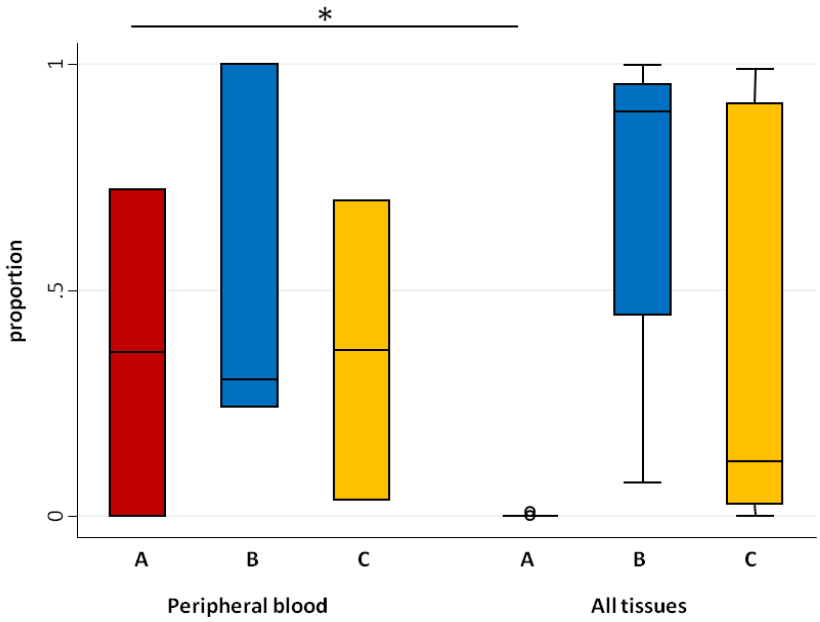


Figure 3.6. Transcript abundances of *var* gene groups in parasites found in the tissues versus peripheral blood. Transcripts are shown as proportions of the total number of *var* gene group transcripts for parasites found in each tissue of each patient. The box plots outline 25th and 75th percentiles, with the median indicated as a line inside each box and the 5th and the 95th percentiles are illustrated by the whiskers. Outliers are indicated by the open circles. (ANOVA; *$p \leq 0.05$ after Bonferroni correction).

A major drawback for this sub-study on matched tissues and peripheral blood is the sample size. Because of the difficult nature of post-mortem studies and the imminent end of the parent study, we were not able to recruit more patients. For this reason, the data lacks statistical power. Apart from that, we experienced a lot of problems with the blood samples, which thankfully our collaborators discovered to produce poor quality RNA perhaps due to drug contamination, circulating toxins and other material released from dead parasites. We have struggled to get consistent and meaningful results in trying to use it in our genotyping and *var* transcription analysis which makes the results from this data set unreliable.

3.5 DISCUSSION

Cerebral malaria is one of the most dangerous and often fatal complications of *P. falciparum* infections in African children. Over the years, much information on the relationship between clinical malaria and *var* genes has been collected using *in vitro* and molecular techniques. The present study aimed to analyse the proportion of transcripts belonging to *var* group A, B, and C present in various tissues and peripheral blood using the unique resources of the clinicopathological study of fatal paediatric malaria in Blantyre, Malawi. We used qRT-PCR to measure *var* gene expression in pRBC sequestered in the brain, heart and gut in children who died from cerebral malaria and children that died from other causes with a concurrent *P. falciparum* infection.

As expected, the quantification of the proportion of *var* gene groups in gDNA indicated similar distributions to the 3D7 genome in the parasites from different clinical presentations and tissues. Therefore we conclude that the differences observed in *var* group expression were due to transcriptional regulation during symptomatic malaria or due to receptor regulation that then "chooses" certain pRBC expressing particular transcripts to sequester, and not primer bias or DNA composition. Our results are directly comparable to those from equivalent field studies on peripheral blood and extend these observations to sequestered populations of pRBC. In the *var* transcription analysis, we found major differences in *var* group transcription between CM1 and CM2 patient isolates, with *var* group B being significantly more highly expressed in CM1. However, we observed no significant differences in the proportions of *var* gene groups between isolates found in CM2 patients and parasitaemic controls. Group A was

prominent in both these patient groups, with group B being the least expressed regardless of tissue.

The infecting parasites in CM1 patients showed quite distinct *var* expression, the proportion of *var* group B transcripts was higher compared to CM2 and PC patients and group A were down-regulated, particularly in the brain and gut. The number of children in CM1 (n=5) was the similar as in the PC group, thus offering the same analytical power for comparison and yet the CM1 expression profiles resemble the overall genomic 3D7 percentages. This result clearly indicates that there are other factors that are influencing *var* gene expression in CM1. It is possible that CM2 and PC express a specific subset of *var* genes that are skewed from the overall genomic set because either 1) the parasites have a specific transcriptional regulation pattern, or 2) parasite transcription is not skewed away from the genomic – but a subset of parasites are chosen to bind based on receptor expression on the endothelial cells.

One of the clues to the unique *var* expression in the CM1 group was HIV status of the patients considering that the majority of the patients, 80% (n=4) were HIV positive. Unfortunately, the total number of patients within the group was small and lacked statistical power to perform within-group comparisons. However, we were able to compare total HIV positive individuals (n=8) versus HIV negative individuals (n=12) and *var* gene group A proportional expression was high in the HIV- patients while group B were upregulated in the HIV+ patients. This confirms our findings that the up-regulation of group B *vars* in the CM1 group might be influenced by their HIV status. The HIV influence on *var* gene expression was hinted even in the small number of

children in which we were comparing *var* expression between tissues and peripheral blood. Although there was no significant difference in *var* group B expression between the HIV- and HIV+ patients, group B was significantly expressed in the HIV+ patients compared to group C.

The differences in vascular pathology between CM1 and CM2 are striking. In CM1 cases, patients have a heavy sequestered load of pRBC in brain microvasculature but no haemorrhages, thrombi or other vascular pathology. In contrast, patients with the CM2 variety of cerebral malaria show the same levels of sequestration and have significant vascular pathology (Taylor, Fu et al. 2004). In adults, HIV infection has been found to be associated with severe forms of malaria and increased risk of CM (Chirenda, Siziya et al. 2000; Chirenda and Murugasampillay 2003; Grimwade, French et al. 2004; Kamya, Gasasira et al. 2006). On the contrary, another study in Zaire showed that there was no differences malaria incidence between children with progressive HIV-1 infection and those who were HIV (Greenberg, Nsa et al. 1991).

In this study, all the children were similar in age and there was no difference in parasitaemia at admission or death in all the diagnostic groups. This is in agreement with the trend observed in the main clinicopathology study where there is generally no association between parasite density and disease severity. Our results present strong evidence that HIV plays a role in influencing on *P. falciparum var* expression. Therefore, it is possible that HIV infected children are at risk of developing cerebral malaria because of an increase production of particular molecules implicated in both diseases such as nitric oxide synthase, ICAM-1 and VCAM-1 (Mannick, Stamler et al. 1999). In

this case, the upregulation of *var* gene group B in CM1 infections may be a result of altered host response encompassing elevated levels such endothelial receptors and immunosuppression, considering division of the samples by HIV reactivity showed a similar upregulation of *var* gene group B genes although *var* gene group A remained highly expressed in HIV negative cases.

Expression of both *var* group A and B genes have been found to be associated with severe malaria (Kyriacou, Stone et al. 2006; Rottmann, Lavstsen et al. 2006; Kalmbach, Rottmann et al. 2010; Avril, Tripathi et al. 2012; Lavstsen, Turner et al. 2012) which could imply that both these *var* gene classes are favourable in binding to endothelial receptors of the host and therefore likely to be involved in sequestration of pRBC in the brain and other organs. Recently, other independent work has confirmed the up regulation of domain cassette 8 (DC8) *var* genes by parasites adhering to human brain endothelia (Lavstsen, Turner et al. 2012). This is also consistent with the finding of Claessens et al (Claessens, Adams et al. 2012) and Avril et al (Avril, Tripathi et al. 2012) which supports the idea that parasites expressing DC8 and DC13 var genes have a high potential to cystoadhere to brain endothelia and other tissues.

The lack of variation in group *var* expression between organs in any diagnostic group was unexpected. Histological studies on fatal malaria in South East Asian adults have shown few differences in endothelial receptor expression levels between organs, except for CD36 which was sparse on brain microvasculature, although heart and gut were not specifically examined in these studies (Turner, Morrison et al. 1994; Silamut, Phu et al. 1999). A study on paediatric malaria in Ghana also showed staining for ICAM-

1, VCAM-1 and E-selectin in association with pRBC in brain microvasculature of fatal CM patients (Armah, Dodoo et al. 2005). Both adult studies showed limited upregulation of endothelial receptors compared to non-malaria controls apart from ICAM-1 and E-selectin. This may account for the homogeneity of *var* expression at group level across organ sites and in both CM2 and PC diagnostic groups, and for the high proportion of upsA genes which are more likely to contain the DBLβc2 domain combination, previously shown to mediate adhesion to ICAM-1 (Smith, Craig et al. 2000).

An alternative explanation for the same *var* types being commonly observed may be due to certain *var* genes being preferentially transcribed, either due to switching at rapid on-rates, slow off-rates, or both. This phenomenon is reviewed in detail in (Gatton, Peters et al. 2003). If certain *var* antigens are more likely to be expressed by an individual parasite, and there is no specific selection for *var* antigens at the tissue, then they will by consequence be more likely to be detected in studies such as these. In *P. falciparum* research, post-mortem tissues and true animal models of sequestration are scarce. The findings suggest a similarity in antigen expression between tissue and peripheral blood. It has been shown that the same circulating parasites end up sequestering in the different organs, hence peripheral studies may be indicative of the sequestering parasite population (Montgomery, Milner Jr. et al. 2006). However, *var* expression in the human host is massively diverse and tissue-specific *var* gene expression occurs (Montgomery, Mphande et al. 2007) and yet it is the same group expression is observed despite different pathologies between CM2 and PC. This

could suggest that *var* gene expression is indeed structured and hierarchical (Discussed in Chapter 1) and the possibility of *var* gene selection depending on the environment and/or demand of affinity of receptor present in that particular tissue cannot be ruled out. With 60 variant genes encoding the expression PfEMP1, antigenic variation generates a wide antigenic repertoire (See Chapter 1 for details). The *var* groups are broadly classified into groups and subgroups; therefore, it could be that different genes belonging to the group are expressed. Perhaps these groups should be placed into smaller classification based on structure such as the Lavstsen system for easy identification and comparison.

Susceptibility to severe forms of malaria is attributed to a combination of both parasite, through sequestration, and host factors, through induction of the immune system (discussed further in Chapter 4). Therefore, what should also be taken into consideration when performing such investigations is a complete knowledge of the expression pattern of these host dependent factors specifically triggered during a clinical episodes that might play an equally important role in parasite sequestration as they could be used in the development of prognostic test that could be used to predict the outcome of *P. falciparum* infections (Oakley, Kumar et al. 2007; Oakley, McCutchan et al. 2008).

The potential influence of HIV and sample size has already been pointed out as one of the drawbacks of this study, such that the results should be interpreted with caution. Nonetheless, in their study Lavstsen *et al.* 2012, indicates that parasites expressing specific *var* genes mainly belonging to group A and B are likely responsible

for SM and therefore host immune system is likely to recognise these *var* genes first. This may be a result of particular adhesive domains binding to endothelial receptors with high affinity, and warrants further investigation of the full length sequences of some of the dominant antigens in order to identify their different binding domains, which can further be used in adhesion assay to identify host molecules they interact with.

3.6 CONCLUSION

In conclusions, the present findings suggest that different *var*/PfEMP1 may influence disease pathologies of cerebral malaria. These results also suggest that there might be a certain order in *var* gene transcription, in agreement to what was described by Recker and others (Recker, Buckee et al. 2011), that predicts similar *var* gene expression in the tissues regardless of pathology. This is also the first time, to our knowledge, to show an influence of HIV status on *var* gene repertoire of sequestered parasites. The question that remains is whether HIV is affecting *P .falciparum* infection dynamics at the level of the host response, transcription level or host selection level. Although patient HIV status may have been overlooked in previous field *var* gene studies, it should be something that should be considered in the future. As we have seen from our data, host response in HIV positive and HIV negative individuals are quite different.

Chapter 4

4. COMPARISON OF HOST RECEPTOR EXPRESSION AND CYTOKINE PRODUCTION IN MALARIA AND HIV CO-INFECTION

4.1 INTRODUCTION

The sequestration of parasitised erythrocytes in the microvasculature of vital organs is central to the pathogenesis of severe falciparum malaria. Mature trophozoites and schizonts are removed from the peripheral circulation (Bignami and Bastianelli 1889), due to adhesion of the infected erythrocytes (Miller 1969) to the host receptors on the surface of vascular endothelial cells, leading to sequestration of the pRBC in the tissues. The consequences of pRBC cytoadhesion and sequestration to disease pathology and the host cells receptors involved have already been emphasised in Chapter 1.

The parasite-host interactions described above illustrate a complex web, with clinical isolates having a plethora of host receptors to adhere to. Some vascular receptors such as CD36 are common, present on varied cell types and expressed at all times in a wide range of vascular beds. Such receptors are regarded as constitutive and their expression is not related qualitatively or quantitatively to severity of disease. Other receptors such as ICAM-1 and E-selectin are inducible (Berendt, Simmons et al. 1989), their expression is modulated by local environmental factors such as inflammatory cytokines (Cotran, Pober et al. 1988; Ketis, Lawler et al. 1988; Wellicome, Thornhill et al. 1990) and their upregulation in cerebral vessels of CM patients suggest

involvement in cytoadhesion (Turner, Morrison et al. 1994; Brown, Hien et al. 1999). Whilst the distribution of these receptors in human tissues is not uniform, reflecting the phenotypic and functional heterogeneity of the endothelia (Kuzu, Bicknell et al. 1992), some receptors such as ICAM-1 and CD36 work together through synergistic behaviour to increase overall binding efficiency (McCormick, Craig et al. 1997). Such synergic traits significantly contribute to increased cytoadhesion, especially endothelial receptors that do not appear to be up-regulatable (Petzelbauer, Bender et al. 1993).

Limited post-mortem studies have been used to investigate sequestration and the expression of cytoadherence receptors *in vivo* (Turner, Beckstead et al. 1987; Ockenhouse, Tegoshi et al. 1992). These have demonstrated the widespread endothelial activation that occurs in fatal malaria and the major receptors involved in brain sequestration. So far, such studies have only been performed in adults. Considering that the major burden of disease occurs in children who have limited immunity against the parasite, and the inflammatory response can have a profound effect on protein expression, receptor upregulation might be different in children.

CM pathology is also attributed to local cytokine release that potentially contributes to organ specific pathophysiology as seen in the brains of fatal CM patients (MacPherson, Warrell et al. 1985; Pongponratn, Riganti et al. 1991; Turner, Morrison et al. 1994; Seydel, Milner et al. 2006; Milner, Valim et al. 2012). There is compelling evidence that the inflammatory responses seen in the brain in cases of CM involve an increased systemic production of pro-inflammatory cytokines, especially TNF, lymphotoxin, IFN-γ and interleukin 1β (IL-1β) (Pober 1988). Cytokine overproduction

leads to the upregulation of ICAM-1, VCAM-1 and E-selectin by cerebral endothelial cells. This in turn facilitates the adherence of pRBC in blood vessels (Armah, Dodoo et al. 2005).

In sub-Saharan Africa the high burden of malaria is an important risk factor for other co-infections such as HIV in adults, malnutrition in children and non-typhoid salmonellae (NTS) (reviewed in (Feasey, Dougan et al. 2012)). In African adults and children, invasive strains of NTS have emerged with an associated fatality of 20-25% (Feasey, Dougan et al. 2012). NTS co-infection has been associated with high malaria mortality (Berkley, Bejon et al. 2009). The association of NTS infection with hemolysis in SMA patients is well established (Mabey, Brown et al. 1987; Bronzan, Taylor et al. 2007) and in murine malaria models (Cunnington, de Souza et al. 2010; Roux, Butler et al. 2010).

Parts of the world with high malaria rates also carry a high burden of HIV (reviewed in (Hochman and Kim 2009; Hochman and Kim 2012)). Many aspects of the relationship between malaria and HIV remain unanswered (Hochman and Kim 2009; Hochman and Kim 2012). While it has been shown that malaria infection increases HIV viral load (Kublin, Patnaik et al. 2005), it has yet to be demonstrated if this translates to higher rates of HIV transmission among populations, even though it has been shown that HIV predisposes to frequent episodes of symptomatic malaria in children and adults (Grimwade, French et al. 2004; Cohen, Karstaedt et al. 2005; Kamya, Gasasira et al. 2006).

Table 4.1. Cytokine production in HIV and Malaria infection.

Cytokine	HIV [a]	Malaria [b]
TNF-α, TNF-β	↑ MDM, U1	↑associated with CM* ↑parasitaemia = anaemia
IL-1β	↑ MDM, U1	↑ associated with CM*
IL-6	↑ MDM, U1	↑ associated with SM*
IL-10	↓ MDM, U1	↑ associated with SM* =parasitaemia, anaemia ↓CM
IL-13	↓ MDM, ↑ MDM	
IL-16	↓ MDM	
IL-12		↑associated with SM* ↓parasitaemia
IL-4	↓monocytes, MDM, ↑monocytes, MDM, PBMC, U937	↓parasitaemia =parasitaemia, anaemia, CM
IFN-γ	↓monocytes, MDM ↑U1, U937	↑ associated with SM* ↓parasitaemia = anaemia

[a] cytokine data generated using *in vitro* techniques, reviewed in (Kedzierska, Crowe et al. 2003); [b] cytokine data generated using mouse model, reviewed in (Angulo and Fresno 2002); * cytokine data generated from humans, reviewed in (Hochman and Kim 2009). MDM, monocyte-derived macrophage; DC, dendritic cells; U1 and U937 are MDM cell lines

Co-infection of falciparum malaria and HIV seems to be mutually detrimental; HIV increases the risk of clinical malaria with the greatest impact in immune suppressed individuals. HIV infects and depletes CD4+ T lymphocytes, putting patients at risk for opportunistic infection and malignancy. Like malaria, control of the HIV infection and pathogenesis is regulated by pro-inflammatory cytokines. As a potential means of affecting disease course and outcome in other infections such as malaria, HIV affects the systemic inflammatory response, causing activation and/or immune cell apoptosis as well as pro-inflammatory cytokine (Table 4.1) and chemokine elevation in the plasma and lymph nodes (reviewed in (Hochman and Kim 2009; Hochman and Kim 2012)). HIV dysregulates cytokine production pathways including decreases in pro-inflammatory cytokines IL-12 and IFN-γ and increases in anti-inflammatory cytokines such as IL-10 (Yadav, Fitzgerald et al. 2009). In addition to cytokines including TNF, IFN-γ, IL-1β, and IL-6 are increased in brain tissue and cerebrospinal fluid (CSF) of patients with Acquired Immunodeficiency Syndrome (AIDS) (Buckner, Luers et al. 2006).

In malaria infections, TNF-inducible cell adhesion molecules are up-regulated, providing pRBCs, uninfected erythrocytes, leucocytes and platelets increased substrates for potential adherence when they are expression of the appropriate receptors (see Chapter 1). Host platelets and mononuclear leucocytes appear to be involved in the pathogenesis of both human and murine CM (Lou, Lucas et al. 2001). Thus, through inflammatory mechanisms described above, HIV can influence the upregulation of adhesion molecules on endothelial cells, which may compound the adherence and sequestration of pRBC. However, the full extent to which this

inflammatory response in HIV infections exacerbates pRBC adherence and sequestration is not known. The previous chapter considered the possibility of HIV altering the host inflammatory response, such that changes in endothelial receptor expression in turn affect antigen expression in malaria. This chapter uses RT-qPCR to analyse post-mortem tissues to ascertain and compare the expression of host genes involved in apoptosis, cytokine production and endothelial receptor expression in malaria and HIV co-infections.

Objectives

i. To determine the expression of putative sequestration receptors and cytokines in the brain, heart and gut from fatal cases of *P. falciparum* paediatric malaria
ii. To compare the distribution of putative receptors and cytokines in paediatric *P. falciparum* patients with different clinical presentations of cerebral malaria
iii. To compare receptors and cytokines distribution between HIV and *P. falciparum* co-infections versus *P. falciparum* infections alone

4.2 RESULTS

4.2.1 Clinical and diagnostic details

This project utilised clinical samples collected under the parent clinicopathological study of fatal paediatric malaria and a summary of the patients' clinical details are given in Table 4.2. The full description of the clinical samples and all methods has been given in Chapter 2.

Table 4.2. Summary of the clinical details of patients in main study.

Diagnosis	Age[a]	HIV+	Time to death[b]	Time to autopsy[b]	Admission parasitaemia[c]	Final parasitaemia[c]
CM1	75.6 ± 40.6	4/5	20:58 ± 16:55	07:49 ± 03:06	382,990 ± 07,769	66,142 ± 120,250
CM2	61.6 ± 49.4	3/11	09:32 ± 09:56	07:15 ± 06:26	247,837 ± 34,794	210,011 ± 350,186
PC	34.3 ±23.3	1/4	13:34 ± 07:32	10:01 ± 05:20	87,624 ± 57,274	79,668 ± 78,700

[a] months, [b] hours:minutes, [c] parasites/μL in peripheral blood are given as means for all patients in each diagnostic group

The overall mean age of the children was 57.2 ± 37.8 months and there were no differences in parasitaemia at admission or at death, or in the length of time to autopsy (Chapter 3, section 3.4.1). Patients were recruited from four different malaria seasons between 1999 and 2004. However, because of limited resources, only about half of the

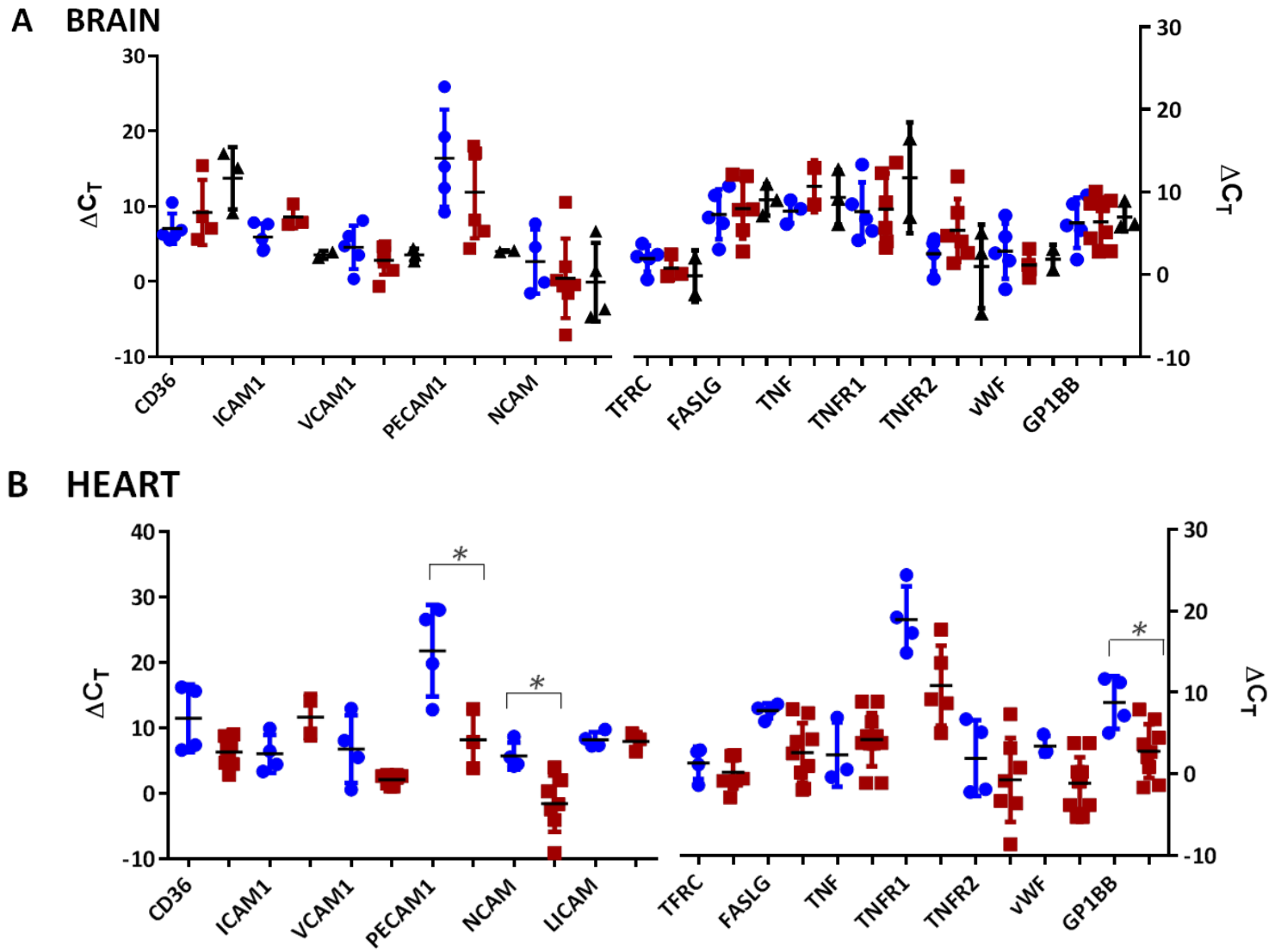
patients were selected for this sub-study from the cohort and not all three tissues were matched for all patients selected in this analysis.

Table 4.3. Clinical details of patients

Case no.	Diagnosis	Tissue	HIV status
CM1	**Sequestered parasites in the brain**		
38	Clinical CM*	brain, gut	-
74	Clinical CM	heart	+
84	Clinical CM	brain, heart, gut	+
79	Clinical CM	heart, gut	+
CM2	**Sequestered parasites in the brain and associated pathology**		
28	Clinical CM	brain, heart, gut	-
55	Clinical CM	brain, heart, gut	-
60	Clinical CM	brain	-
61	Clinical CM	brain, heart, gut	-
62	Clinical CM	brain	-
63	Clinical CM	brain	-
78	Clinical CM	brain, heart, gut	-
PC	**Parasitaemic Controls**		
31		brain	+
65	Coma of unknown cause with hypoglycaemia- Reye's	brain	-
77	Gangrenous bowel	brain	-

*CM cerebral malariA

4.2.2 Comparison of receptor expression and local cytokine release in the diagnostic groups



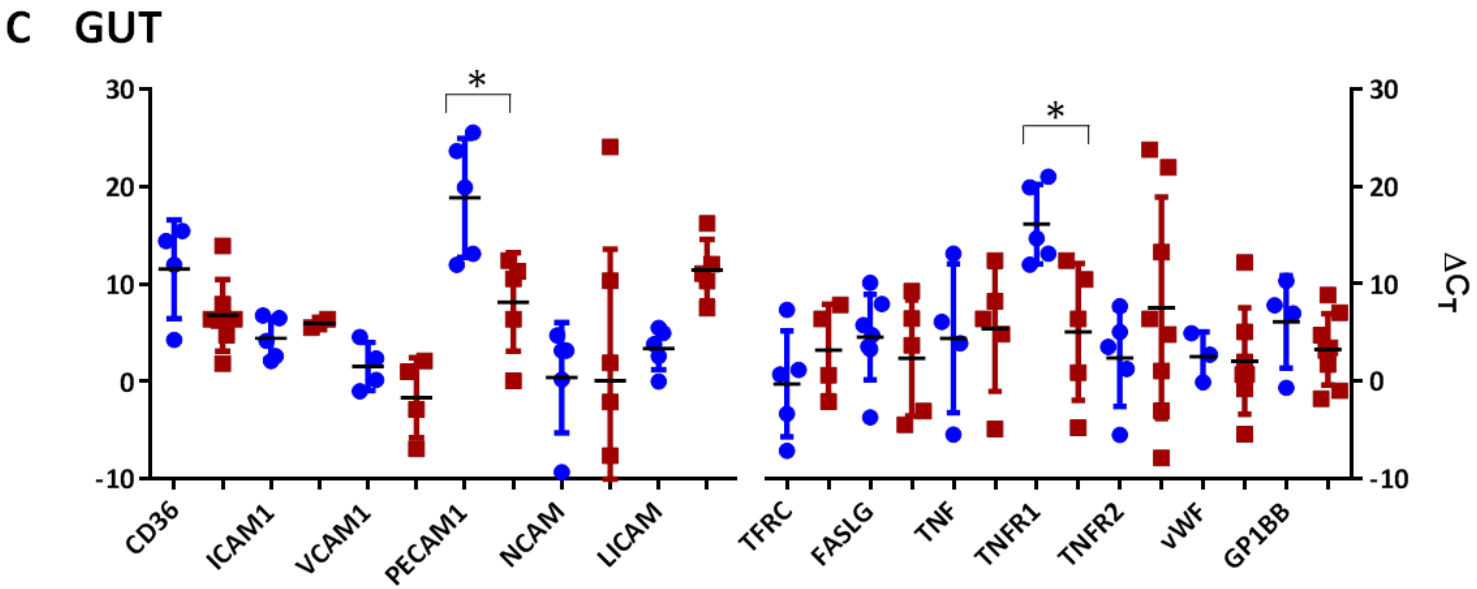


Figure 4.1. Transcript abundance of each cytokine or receptor in the post-mortem tissues of parasitaemic children. Description for each cytokine or receptor is given in Table 2.4. Transcript abundances for the brain (A), heart (B) and gut (C) are shown according to the diagnostic groups; Blue is CM1 (n=4), Red is CM2 (n=7) and Black is parasitic controls (n=3). The tissue available for each patient that was included in the analysis is listed in Table 4.3. Each ΔC_T value was calculated relative to the average of housekeeping gene GAPDH. The mean with a standard deviation is indicated by the cross lines (ANOVA; *$p \leq 0.05$ and **$p \geq 0.001$ after Bonferroni correction).

Comparison of production and expression of 12 selected cytokines and receptors (Chapter 2, Table 2.4) in the clinically different CM diagnostic group was conducted using relative quantitative PCR with glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an enzyme that catalyzes the breakdown of glucose for energy and carbon molecules, as an endogenous control. Data analysis was statistically challenging because of the small sample size in each diagnostic group.

Surprisingly, most genes, with exception of ICAM-1 and TNF, were more highly expressed in the heart and gut of CM1 than CM2. CM1 group had the most patients co-infected with HIV and therefore possessed a degree of immune suppression, the level of which unfortunately is unknown in these patients. There were no significant differences in expression of the receptors and cytokines between the diagnostic groups in the brain. Although, there is an indication of elevated ICAM-1 in CM2, the sample size was too small to draw solid conclusions. This could also imply that the physiological responses to malaria infection are similar in these patients, irrespective of severity of disease. Both CM1 and CM2 are severe (and in these cases, fatal) forms of malaria and inflammatory responses to pRBC occur even in the parasitaemic controls. I have already pointed out the possibility of the presence of HIV in some of these patients potentially influencing cytokine release, and in turn receptor expression (discussed in Chapter 3). Because of the difficulty of the nature of post-mortem sample collection, there were not enough PC to be included in this analysis for the heart and gut. In spite of this, we observed some tissue and/or diagnostic group cytokine/receptor differences. There was an indication of CD36 elevation in CM1 and ICAM-1

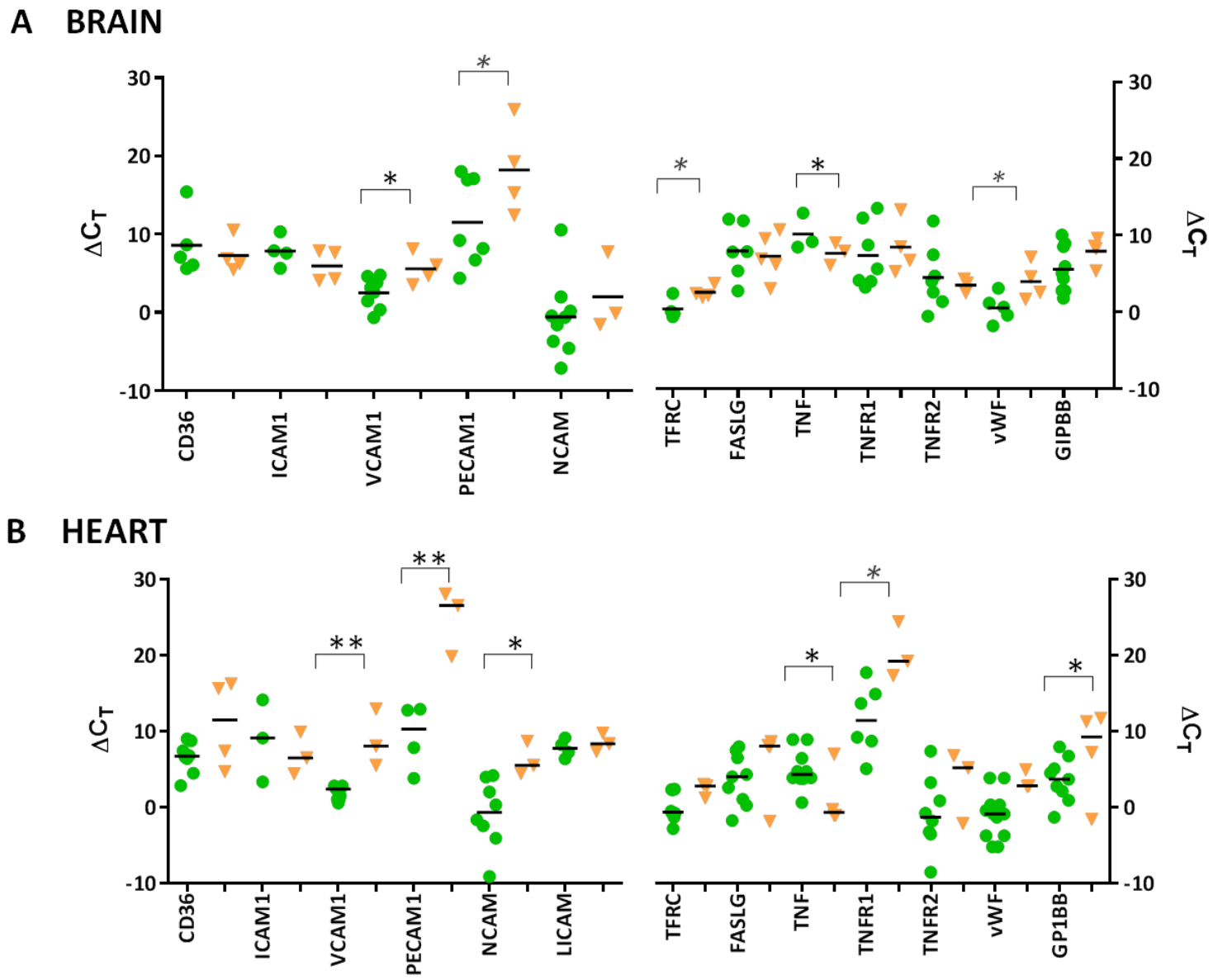
upregulation in the heart of CM2 patients. In CM2, the expression of ICAM-1 appeared to be elevated in all the tissues but it was significantly higher in the gut (p=0.002). On the other hand, there was a significant increase of neural cell adhesion molecule (NCAM; p=0.037) and apoptosis gene -1 Ligand/CD95L (FASLG; p=0.059) in the heart of CM1 cases. NCAM has been recently identified to facilitate *P. falciparum* binding but the parasite ligand for this receptor is not known (Pouvelle, Matarazzo et al. 2007). FASLG is a possible indicator of increased production of an apoptosis gene FAS, that also aids in regulation of the immune system.

The cytokine/receptor expression pattern in the gut was very similar to the heart. Three receptors: CD36, PECAM-1and VCAM-1 (p=0.057 in the heart) were elevated in both tissues in CM1 but only PECAM-1 was significantly increased in the heart (CM1 versus CM2; p=0.034 in the heart and p=0.050 in the gut). PECAM-1 and CD36 are widespread on tissue endothelia and other types of cells, and they have also been implicated in pRBC binding (see Chapter 1). Surprisingly, an increase in the expression of glycoprotein Ib/beta polypeptide/CD42c (GP1BB; p=0.034 in the heart) was also observed. This molecule forms a part of the GPIb-V-IX system that constitutes the receptor for vWF and mediates platelet adhesion in the arterial circulation, and an increase in vWF itself. The presence of PECAM-1 indicates endothelial activation and inflammatory responses to the presence of pRBC. These physiological responses also trigger the upregulation of VCAM-1 and vWF which are early markers of endothelial activation, especially in vascular disorders (Zeigler, Rosenfeld et al. 1996; van Mourik and Romani de Wit 2001; Szmitko, Wang et al. 2003; Eikemo, Sellevold et al. 2004).

Interestingly, these genes are associated with platelet-mediated binding of pRBC, and therefore potentially contribute to cytoadhesion (Bridges, Bunn et al. 2010).

Although not statistically significant, there was an increase in TNF expression in the brain and heart compared to the gut (Figure 4.1A and B). In malaria infections, TNF is detected in circulation during the erythrocytic stages of infection (Grau, Piguet et al. 1989; Kern, Hemmer et al. 1989; Kwiatkowski, Hill et al. 1990) and has been shown to have both beneficial and detrimental effects. High levels of this cytokine have been associated with malarial pathology such as fever (Karunaweera, Grau et al. 1992) and CM (Grau, Fajardo et al. 1987; Kwiatkowski, Hill et al. 1990). TNF has also been shown to have anti-parasitic activity by inhibiting blood stage infection of *Plasmodium chabaudi* in mice (Clark, Hunt et al. 1987) and rapid clearance of fever and parasites in humans (Mordmuller, Metzger et al. 1997). However, tumor necrosis factor receptor 1/CD120a (TNFR1), a crucial receptor for TNF-induced neurotoxic processes, was upregulated in the heart and gut which could imply regulation using an alternative pathway.

4.2.3 Comparison of receptor expression and local cytokine release by HIV status



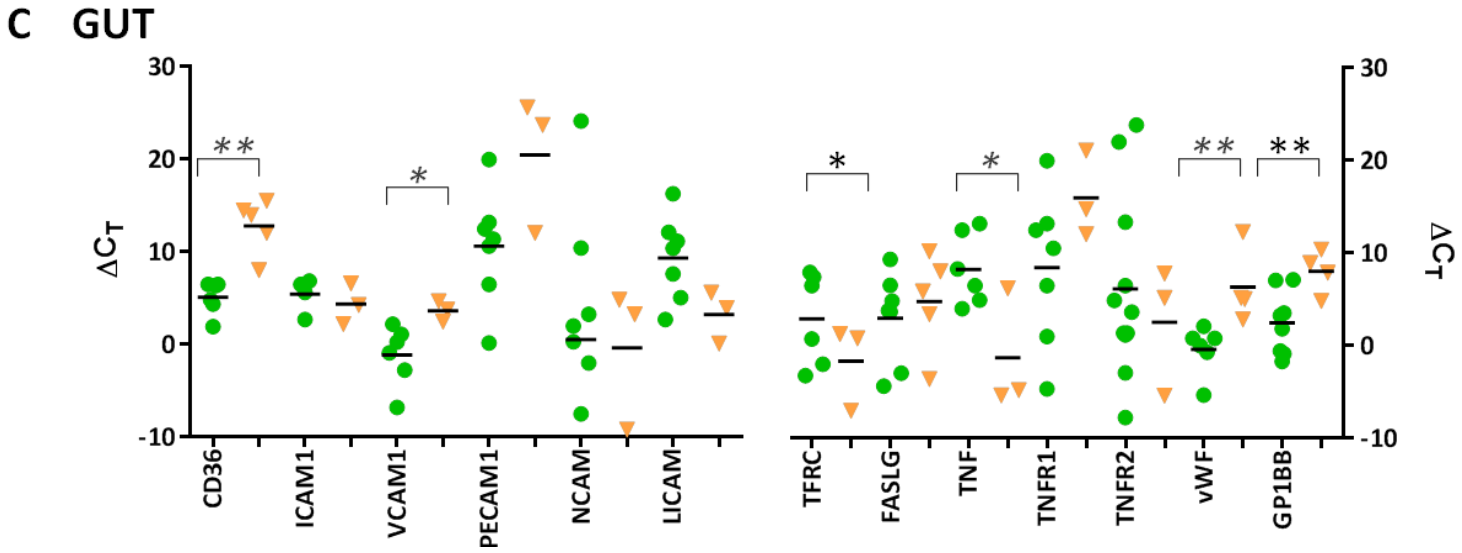


Figure 4.2. Transcript abundance of each cytokine or receptor in HIV positive versus HIV negative children suffering from cerebral malaria. Description for each cytokine or receptor is given in Table 2.4. Transcript abundances for the brain (A), heart (B) and gut (C) are shown according to HIV status; HIV positive in orange also shown by the triangles and HIV negative in green also shown by closed circle. The dash in each sample population represents the mean. Each ΔC_T value was calculated in relative to the average of housekeeping gene GAPDH. (ANOVA; *$p \leq 0.05$ and **$p \geq 0.001$ after Bonferroni correction).

There is some evidence that HIV infection might influence the expression of host receptors which in turn might determine which pRBC can sequester (discussed in section 4.1 and in Chapter 3). In order to explore this possibility, the levels of genes encoding for 12 selected cytokines and receptors (listed in Chapter 2, Table 2.4) that potentially influence HIV and malaria infections were compared against a housekeeping gene between HIV positive and HIV negative cases. Of the 14 children, only 5 were HIV positive (CM1 n=3; CM2 n=1 and PC n=1; Table 3.2). The PC group comprises children that were HIV+ plus another disease that was their cause of death. In order to avoid conflict of interest on malaria effect on cytokines and that of other diseases, only children in the CM2 and CM1 groups were included in this analysis.

The pattern of cytokine/receptor upregulation in the tissues of HIV positive children was generally similar to CM1. This was not surprising as most of the HIV positive children had a CM1 diagnosis (see also Chapter 3, section 3.4.4). Cytokine/receptor gene expression patterns were also similar between the tissues for the respective HIV status groups. CD36 was upregulated in the heart and gut of HIV+ patients, with a significant increase in the gut (p=0.0001). In agreement with data from adults (Meroni, Riva et al. 2005), expression of CD36 increases in HIV-infected children and circulating monocytes regardless of antiretroviral treatment (Meroni, Giacomet et al. 2005).

Genes encoding PECAM-1, VCAM-1, vWF and GP1BB were prominent in the HIV positive group in all the tissues, as was seen in CM1 above. Expression of TNF was

significantly increased in the CM2 cases in all tissues, ($p \leq 0.042$ for all tissues), in agreement with Emmie Mbale and Chris Moxon (personal communication) who showed increased TNF in the sera of HIV negative children with severe malaria infections compared to children with severe malaria and HIV co-infection.

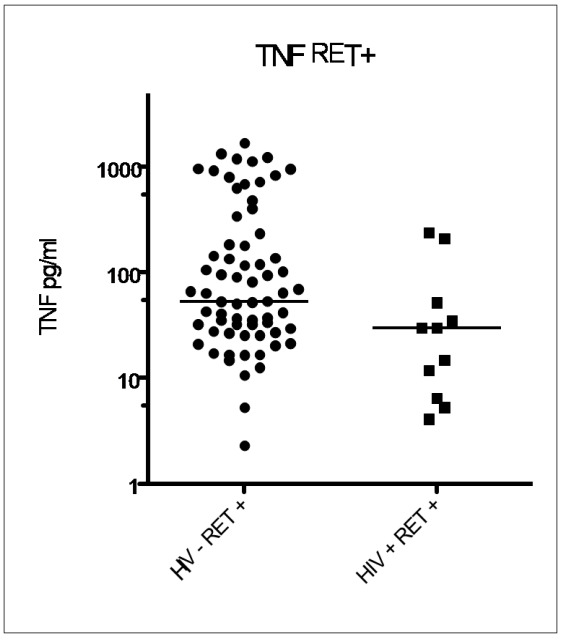


Figure 4.3. Comparison of TNF plasma concentrations between CM children who are HIV-infected and HIV-uninfected. In 80 CM patients with malaria retinopathy, TNF concentrations on admission were higher in 69 HIV negative patients than in 11 HIV positive children: mean 53.7 units/mL vs 29.8 units per mL, respectively (p=0.03) (*adapted from Emmie Mbale and Chris Moxon*)

Some of the observed gene upregulation was localised to a particular tissue or diagnostic group, as in the case of TFRC which was higher in HIV positive cases in the brain while low in the gut of HIV negative children (p=0.041 and p=0.031 respectively). While the upregulation of NCAM and TNFR1 was similar in the brain and gut of both

groups, it was significantly raised in the heart of HIV positive children (p=0.049 and p=0.016, respective of gene). GP1BB was high in the gut of HIV positive children (p=0.007) while NCAM and IFNG were also elevated in children in this group.

4.3 DISCUSSION

Despite the high mortality associated with CM, an understanding of the underlying pathogenic mechanisms has been slow to evolve and there are still grey areas requiring investigation. Some of these areas are unable to be even roughly investigated, because post-mortem samples from patients in whom CM was established prior to death are rare. Over the years, several hypotheses on the parasite and the host side have been developed to explain the pathogenesis of CM. The release of plasmodium toxin (glycosylphosphatidylinositol), systematic and local cytokine production, the upregulation of adhesion molecules by tissue endothelia, and the consequences of sequestration i.e mechanical vessel blockage, ischemia, acidosis haemorrhage and excess production of nitric oxide have all been implicated in CM pathology (Newton and Krishna 1998).

Areas with high rates of malaria also carry a heavy burden of HIV (reviewed in (Hochman and Kim 2009; Hochman and Kim 2012)). Initial cross-sectional population studies to determine co-infection dynamics found no significant differences in incidence or severity of malaria between HIV-infected and uninfected individuals (Chandramohan and Greenwood 1998). However, recent work suggests otherwise, that

during co-infection symptomatic malaria incidence and the severity of illness increases (reviewed in (Hochman and Kim 2012)). Both infections are influenced by pro-inflammatory cytokines in control and pathogenesis; however, they differ in the mechanisms by which they respond to physiological changes in their host environment (discussed in Chapter 1) and it is not known if the inflammatory response initiated by HIV affects adherence, sequestration, or disease severity in malaria.

In the previous chapter of this thesis, some evidence was provided on how HIV might "influence" *P. falciparum* variant surface antigen expression. This chapter offers some support to what has previously been speculated, that the induction of adhesion molecules on endothelial cells through inflammatory cytokine release may compound pRBC adhesion and sequestration in malaria, thus intensifying CM pathology. In this study, the expression of selected cytokines and receptors (Chapter 2, Table 2.4) was quantified in the brain, heart and gut obtained from pathologically defined cases of fatal CM and non-cerebral paediatric malaria. The CM patients were further grouped based on their HIV status in order to permit an extensive evaluation of the role HIV plays in paediatric CM on the local production of cytokines and adhesion molecules in different tissues. Due to the difficulties of obtaining post-mortem samples, a small tissue size for the different patient subgroups poses puts this study at a disadvantage. Therefore, this study is mainly descriptive as there was not enough tissue to allow extensive and exhaustive analysis.

Firstly, comparison of cytokine/receptor expression in the CM diagnostic groups confirms what previous studies have shown, that cytokine/receptor activation occurs in

both fatal malaria and non-cerebral malaria and is tissue-specific. We speculate that this might depend on how function of a particular tissue is affected by disease. Several studies in both children and adults have already shown how HIV influences malaria prevalence and epidemiology (reviewed in (Hochman and Kim 2009).

The major variant findings are individually discussed below:

4.3.1 Adhesion molecules

CD36

HIV also alters fat metabolism and distribution regardless of antiretroviral treatment (Dube, Stein et al. 2003). CD36 is one of the receptors implicated in lipodystrophy and plays a crucial role in the cellular uptake and metabolism of lipids and sugars (Aitman, Glazier et al. 1999). Increasing CD36 expression would seem beneficial to the virus as it uses lipids for propagation. However, it may be detrimental to the host as in HIV infected individuals fat distribution is impaired regardless of antiretroviral treatment and the upregulation of CD36 may contribute to pRBC sequestration.

The role of CD36 in CM is questionable considering that brain endothelium does not express CD36 and recently, pRBC binding to CD36 was associated with UM (Ochola, Siddondo et al. 2011). It is proposed that the source of CD36 in the brain is platelets and inflammatory cells, especially monocytes and leukocytes that have been shown accumulated in brain tissue from CM paediatric patients at autopsy (Wassmer, Lepolard et al. 2004).

PECAM-1

PECAM-1 is a glycopeptide adhesion molecule implicated in pRBC-platelet adhesion (Treutiger, Heddini et al. 1997; Heddini, Chen et al. 2001) and was elevated in the tissues of HIV positive patients (Figure 4.1). This is in agreement with data from (Eugenin, Gamss et al. 2006) that showed elevated levels of soluble PECAM-1 in the sera and brain tissue of individuals with HIV encephalitis. Therefore, PECAM-1 upregulation may not be specific to CM but rather an indicator of brain damage. High levels of PECAM-1 offers an explanation for the accumulation of platelets in the brains of CM patients (Wassmer, Lepolard et al. 2004) and the upregulation of host mononuclear leucocytes and monocytes involved in the pathogenesis of both human and murine CM (Brown, Rogerson et al. 2001; Armah, Dodoo et al. 2005). LFA and PECAM-1 have been shown to increase HIV infection of human umbilical vein endothelia cells (HUVEC) by enhancing HIV absorption (Scheglovitova, Scanio et al. 1995).

The presence of high PECAM-1 in the brain is very significant as this receptor is involved in the migration of monocytes from the vasculature through the blood brain barrier (BBB) into brain tissue. In malaria infections, PECAM-1 on its own has never been correlated with severe malaria (Newbold, Warn et al. 1997; Heddini, Pettersson et al. 2001). PECAM-1 together with CD36 also acts as a binding site for microparticles (small fragments shed from plasma membranes or activated platelets during CM infection) present on platelets (Combes, Taylor et al. 2004; Faille, Combes et al. 2009). Perhaps its action in facilitating cytoadhesion is enhanced by acting synergically with

other receptors as its upregulation also coincides with upregulation of CD36 and VCAM-1 within the same tissues.

Furthermore, the binding of platelet microparticles to pRBC contributes to their cytoadherence to brain microvascular endothelial cells *in vitro*. Sequestration may be further enhanced during HIV co-infection by facilitating the release of platelet microparticles via platelet activation (Wang, Zhang et al. 2011).

VCAM-1

In agreement with (Wolf, Tsakiris et al. 2002), elevated VCAM-1 was observed in all three tissues in HIV-infected children. VCAM-1 binding to pRBC is extremely low and is not associated with malarial disease severity in African isolates (Newbold, Warn et al. 1997). Therefore, in this case VCAM-1 is potentially acting as an early marker for endothelial activation.

ICAM-1

There was a slight upregulation of ICAM-1 in the brain and heart of CM2 patients although the results were not statistically significant. Because ICAM-1 is widely expressed in the brain microvasculature affected by sequestration, it is thought to be the likely main receptor for pRBC adherence in that organ (Berendt, Simmons et al. 1989; Graninger, Prada et al. 1994; Turner, Morrison et al. 1994). Unlike Wolf *et al*. 2002, who found a systemic ICAM-1 increase in HIV infected individuals, significant differences in the expression of ICAM-1 between HIV positive and negative children

were not seen in this study. On the other hand, ICAM-1 binds to PfEMP1 variants containing the DBLβ-C2, which is found in all three main *var* gene groups and very common in group A (Kraemer, Kyes et al. 2007). However, *in vitro* studies using HUVEC infected by HIV have shown that virus replication is arrested by adding monoclonal antibodies to ICAM-1 (Scheglovitova, Scanio et al. 1995), suggesting that ICAM-1 upregulation could promote increase in HIV replication.

4.3.2 Cytokines

vWF

This is another marker for early EC activation triggered by inflammation and liberally found in circulation. It is upregulated when the GP1BB receptor that forms part of the GP1b-V-IX, complex is induced. *In vivo,* vWF biosynthesis is limited to EC and megakaryocytes, which mediate platelet adhesion to the site of vascular injury (Stel, Sakariassen et al. 1985; Ruggeri and Ware 1993). During *P. falciparum* infection, vWF levels are elevated in both CM and UM patients (Phiri, Bridges et al. 2011) and the accumulation of platelets offers the opportunity for pRBC to bind to vWF strings via CD36 (Bridges, Bunn et al. 2010). In accordance with Drouet et al. 1990, we found an increase in both GP1BB and vWF in all three tissues of HIV infected children. There was more vWF in CM1 compared to CM2, implying more vWF release in children with co-infection.

The notion that HIV actually infects EC cannot be ruled out. vWF levels are elevated at all stages of HIV/ AIDS (Drouet, Scrobohaci et al. 1990), an indication of endothelia involvement during HIV infection. The presence of HIV infection brings about endothelial damage and the resulting inflammation stimulates vWF release. During both malaria and HIV infection, the endothelium is chronically exposed to an imbalance of cytokine concentrations and consequently the normal endothelium function may be impaired (Zietz, Hotz et al. 1996). Raised vWF plasma levels do not necessarily imply endothelial damage but merely its activation or stimulation (Pober 1988; Blann, Maxwell et al. 1995). With the lack of vWF release in the tissues, these results provide some evidence that vWF plasma levels correlates to its level of expression on EC.

TNF

TNF plays an important role in the pathophysiology of both malaria and HIV infections. In advanced HIV infections, TNF is essential for virus replication and therefore is upregulated (Griffin, Leung et al. 1991; Naif, Ho-Shon et al. 1994). In contrast, Mbale and Moxon (personal communication) found in a cohort of retinopathy positive children almost two times the amount of plasma TNF in HIV- hosts compared to children co-infected with HIV (Figure 4.3). The patients of this study were children less than 5 years of age with CM confirmed by retinopathy with or without HIV co-infection, also enrolled at the malaria research ward at QECH in Blantyre, Malawi. On average, children with CM and HIV co-infection were older than HIV negative children

with CM (p<0.01). The disease course was prolonged in HIV positive children with CM but their short term outcome was not affected and TNF plasma concentration was higher in CM children who were HIV uninfected, compared to HIV infected CM children. This coincides with the significant increase of TNF in all the tissues in vascular pathology-associated CM2 hosts that were predominantly HIV negative, and reduced TNF production in HIV positive individuals.

In malaria infections, TNF has both detrimental and beneficial effects; detrimental as it has been associated with malarial symptoms such as fever (Karunaweera, Grau et al. 1992) and CM pathology (Grau, Fajardo et al. 1987; Kwiatkowski, Hill et al. 1990); beneficial because it has potent anti-parasitic activity and is associated with rapid clearance of pRBC (Mordmuller, Metzger et al. 1997). However, the role of TNF in malaria and HIV co-infections remains unclear. Although there is high TNF expression in hosts with either HIV or malaria infections, co-infections seem to significantly reduce TNF production. One of the receptors that TNF is known to regulate is ICAM-1 (reviewed in (Schofield and Grau 2005; Marsh and Kinyanjui 2006)), which provides some explanation of a slight increase of ICAM-1 in the brain and heart of CM2 cases (Figure 4.1) and lack thereof in the HIV positive individuals. TNFR1-deficient mice exhibit normal susceptibility to *P. berghei* (Grau, Piguet et al. 1989). Conversely, HIV infections of monocyte-derived macrophages induce TNF secretion through TNFR1 that enhances HIV replication (Esser, von Briesen et al. 1991; Esser, Glienke et al. 1996; Esser, Glienke et al. 1998). In accordance with this, a significant upregulation of TNFR1 was observed in the heart and gut. However, this TNF overproduction must be short-

lived and viral load-dependent because infecting macrophages with HIV show reduction of macrophage ability to produce cytokines, leading to lower TNF levels in the HIV positive patients (Ludlow, Zhou et al. 2012).

Pre-treatment of macrophages with TNF prior to HIV-1 infection results in signalling via TNFR2 or CD120b and leads to inhibition of HIV replication (Herbein and Gordon 1997). On the other hand, TNFR2- deficient mice develop protection against CM (Grau, Piguet et al. 1989). We did not see any significant differences in the transcription of TNFR2 in the tissues or the diagnostic groups and it is not clear what role this receptor plays in the HIV and malaria con-infections, except in this case, to suggest that HIV replication is not inhibited.

TFCR and FASLG

Soluble transferrin receptor concentrations are considered to be a sensitive indicator of iron availability in the tissues (Ahluwalia 1998) and have been used as measure of iron deficiency in anaemia of chronic disease (Ray, Ndugwa et al. 2007). Iron deficiency is suggested to protect against malaria parasitaemia and clinical malaria in young Malawian children (Jonker, Calis et al. 2012). There were no significant differences in TFCR upregulation in the diagnostic groups (Figure 4.1) but in the tissues between HIV-infected and HIV-uninfected children (Figure 4.2). TFCR was up regulated in the brain HIV-infected children and in the gut of HIV-uninfected children, indicating that in this case iron deficiency might not be universal in co-infection but rather tissue

specific. Other examples of tissue specific responses include FASLG, which was only significantly higher in the heart of CM1 patients. In both malaria and HIV infections, FASLG is a marker of increased immune cell apoptosis triggered either from within the cell or by signals that are elicited by binding of extracellular "death ligand" to their death receptor, most of which belong to TNF (Pinti, Nasi et al. 2000). Soluble FASLG is extremely elevated during acute falciparum malaria of non-immune adults and decreases during anti-parasitic treatment, suggesting an association with T cell lymphopnia (Pinti, Nasi et al. 2000). The FAS receptor is known to mediate apoptosis of lymphocytes during acute malaria infection; this is in order to achieve balance between pro-apoptotic and anti-apoptotic mechanisms comparable to the situation in the other infectious disease like HIV (Pinti, Nasi et al. 2000).

Other influential cytokines

One of the clear ways that malaria and HIV interact is through local cytokine release from inflammatory responses due to immune system activities and the up-regulation of adhesion molecules. There is a scarcity of direct evidence for local cytokine release in human tissues from CM patients mainly because of the difficulties in obtaining post-mortem samples. Therefore, most of these studies are descriptive as there are not enough samples for extensive and exhaustive analysis, as was the case in this study. Another influential cytokine that ought to be included in future studies is IFN-γ which is known to increase the binding of pRBCs to EC via PECAM-1, possibly through redistribution of PECAM-1 from endothelial junctions to the cell surface

(Treutiger, Heddini et al. 1997). As already mentioned, PECAM-1 contributes to changes in the BBB permeability and enhanced trafficking of HIV-infected monocytes into the brain. Other essential cytokines and receptors that play key roles in both HIV and malaria infections are: IL-10 (Yadav, Fitzgerald et al. 2009), IL-1β, IL-6 (Pober 1987; Buckner, Luers et al. 2006) and lymphotoxin α would also be worth investigating.

4.4 CONCLUSION

In conclusion, the presented results provide evidence indicating that in paediatric CM the upregulation of adhesion molecules might be further influenced by the induction of cytokines such as TNF. However, there is some indication that the presence of HIV influences patterns of adhesion molecule expression, which may result in the manipulation of the immunopathology associated with CM. It is hoped that future studies will include immunohistochemistry to explore HIV influence on cytokines producing cells in the issues. This analysis is limited in that it is not clear if the gene expression is coming from the tissue (gut, brain or heart) or from monocytes that are in that tissue. More effort should be put in developing models that would allow further investigations of HIV/malaria interactions and responses in immune cells and endothelial cells *in vitro* assays.

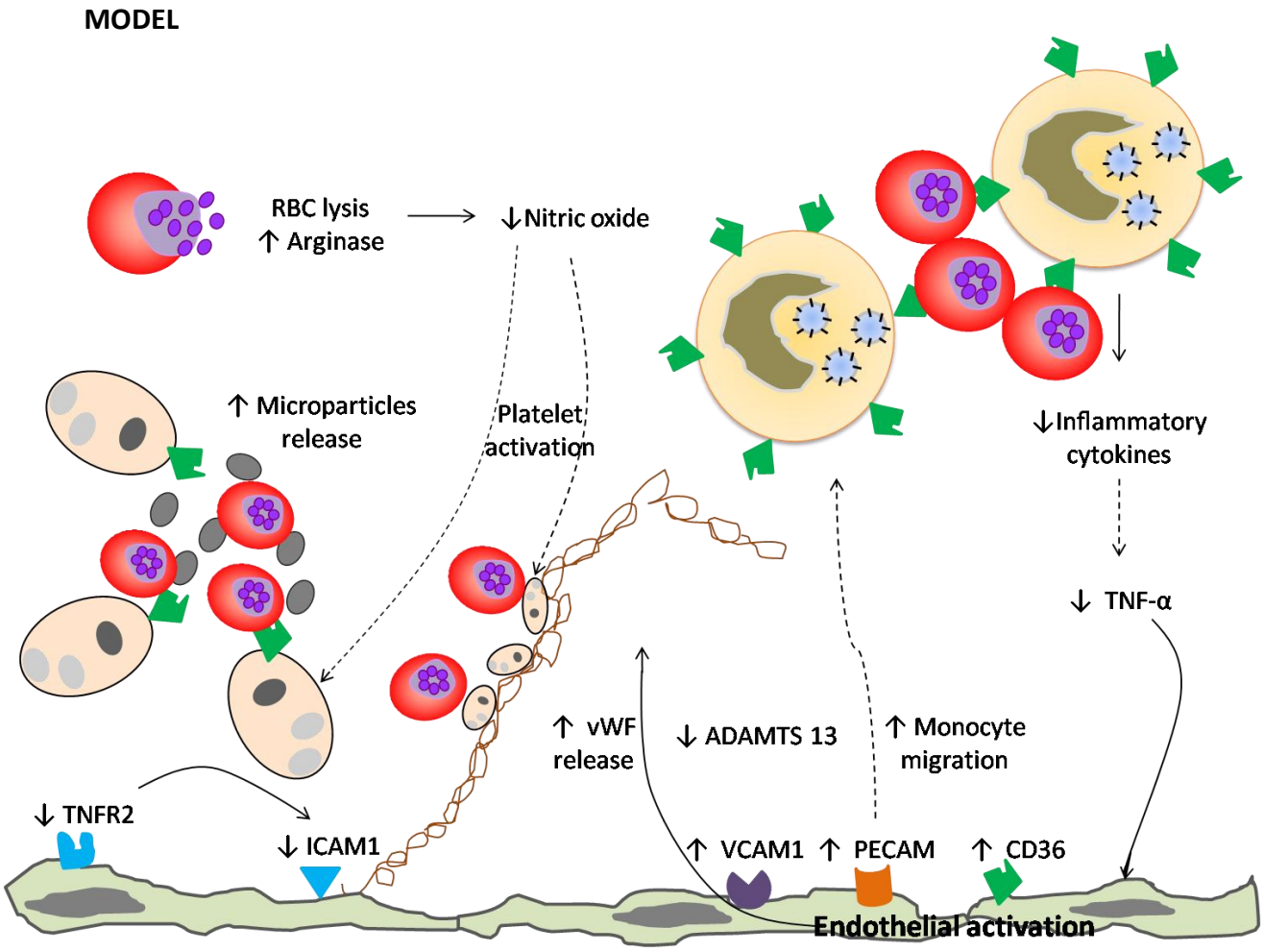


Figure 4.4. Proposed model of the regulation of pRBC cytoadhesion and sequestration in children co-infected with HIV. The presence of both parasites and HIV elicit the immune and inflammatory responses which result in endothelial cell activation and the release of vWF which binds pRBC through platelets. However, production of inflammatory cytokines by HIV-infected macrophages such as TNF is limited. As such, ICAM-1 is down-regulated on the brain endothelia as the TNFR2 receptor that interacts with TNF to upregulated ICAM-1 is also down-regulated. Instead, VCAM-1, PECAM-1 and CD36 are upregulated due to endothelial activation. In the brain, PECAM-1 is used for monocyte migration across the BBB. CD36 is also upregulated on HIV-infected macrophages and potentially mediates pRBC adhesion. The upregulation of arginase due to RBC, depletion of nitric oxide, reduced ADAMTS activity, and platelet activation which causes EC to massively upregulate receptor expression on their surface, and release of microparticles that all provide substrate for pRBC adhesion.

Chapter 5

5. DIFFERENTIAL VAR GROUP EXPRESSION IN PLATELET-MEDIATED CLUMPING ISOLATES OF MALAWIAN PAEDIATRIC MALARIA PATIENTS

5.1 INTRODUCTION

As previously discussed in Chapter 1, one of the most important pathological characteristics of *P. falciparum* infections is the sequestration of large numbers of mature parasites (trophozoites and schizonts) in the microvasculature of vital organs due to cytoadherence of pRBC to host endothelia (Rogerson, Grau et al. 2004). There are several adhesive phenotypes that have been associated with severe outcomes of malaria such as the formation of rosettes, sequestration of pRBC in the brain or placenta (Miller, Baruch et al. 2002), and formation of platelet-mediated clumps (Pain, Ferguson et al. 2001). pRBC adhesive mechanisms that do not utilise endothelia are supported by post-mortem histological studies that have shown pRBC accumulating in the microvasculature (Wassmer, Combes et al. 2006).

A precise role for platelets in malarial pathology is mostly speculative; however, pRBC can form mixed clumps in the presence of platelets *in vitro* in which the platelets act as a bridge between pRBCs. Whether platelet-mediated clumps occur *in vivo* remains unclear but may account for the significant accumulation of platelets described in brain microvasculature of Malawian children who died from CM pathology (Grau, Mackenzie et al. 2003) and thus contribute to microvascular obstruction. Interactions between pRBC and platelets might also lead to platelet activation and release of

inflammatory mediators that upregulate expression of adhesive molecules that facilitate cytoadherence (Srivastava, Cockburn et al. 2008). Platelets are also known to mediate pRBC adhesion to vWF strings (Bridges, Bunn et al. 2010), are believed to have anti-parasitic effects *in vivo* and are able to bind and kill pRBC (McMorran, Marshall et al. 2009).

P. falciparum clumping phenotypes vary between clinical isolates, and the ability to generate clumps in the presence of platelets is significantly greater in CM isolates compared to parasites isolated from uncomplicated malaria patients. Significant associations of the clumping phenotype with severe malaria has been reported in Malawian (Wassmer, Taylor et al. 2008), Kenyan (Pain, Ferguson et al. 2001), Thai (Chotivanich, Sritabal et al. 2004) and Mozambican patients (Mayor, Hafiz et al. 2011), (although not in Malian (Arman, Raza et al. 2007)). The outcomes of these field studies were affected by using different experimental methods to assess platelet-mediated clumping.

Although the molecular binding mechanisms between pRBC and platelets are not fully understood, three platelet receptors have been identified that mediate clumping: gC1qR/HABP1/p32 expressed on the surface of diverse cell types including endothelial cells and activated platelets (Biswas, Hafiz et al. 2007); P-selectin expressed on the surfaces of activated platelets, especially in combination with CD36 (Wassmer, Taylor et al. 2008); and CD36, a glycoprotein receptor expressed on the platelet surface (Pain, Ferguson et al. 2001; Chotivanich, Sritabal et al. 2004). The observation that most field isolates can adhere to CD36 and yet this receptor is not expressed by human

brain ECs has been a mystery, and CD36 was therefore thought to be unimportant in cerebral sequestration. The accumulation of platelets in cerebral vessels suggests that CD36 mediated cytoadhesion in the brain is possible through indirect adhesive events (see also Chapter 4).

The parasite ligand for all the receptors mentioned above is unknown although PfEMP1 is the likely candidate. The parasite ligands for CD36 binding are PfEMP1 variants (Baruch, Pasloske et al. 1995; Baruch, Gormely et al. 1996) encoded by two major subtypes of *var* genes; groups B and C (Robinson, Welch et al. 2003). Binding is mostly via the most N-terminal CIDR domain (Baruch, Ma et al. 1997; Smith, Kyes et al. 1998; Miller, Hudson-Taylor et al. 2002). CIDRα-domains bind to CD36 whereas CIDR-α_1, β and γ domains do not. The CIDR1 of *var* gene groups B and C (full description in Chapter 1) are the CIDRα type and therefore their *var*/PfEMP1 variants bind to CD36. In contrast, CIDR1 domains of *var* gene group A (full description in Chapter 1) are mainly CIDR-α_1 and their PfEMP1 variants are known to not bind to CD36 (Robinson, Welch et al. 2003).

DBLα is another domain of the PfEMP1 that has lectin-like properties that enables binding primarily to RBC bearing A and B blood group oligosaccharides, and to other glycosylated proteins such as CD35 (CR1) and heparin sulphate-like glycosaminoglycans (Baruch, Gormely et al. 1996; Miller, Hudson-Taylor et al. 2002). Cerebral malaria has been linked to DBLα_1 expression, rosette formation and adhesion to ABO blood group antigens (Kyriacou, Stone et al. 2006). Blood type A or B antigens are also implicated in pathology through formation of large rosettes and at higher

frequencies compared to type O, and the fact that rosette formation correlates with transcription of lectin-specific binding domains of PfEMP1.

This chapter is intended to identify specific *var* gene groups involved in platelet-mediated clumping using *P. falciparum* isolates from the peripheral blood of Malawian paediatric malaria patients.

Objectives

i. To determine which particular *var*/PfEMP1 subtypes mediate platelet-mediating clumping

ii. Identify ABO blood groups associated with platelet-mediated binding phenotypes

5.2 HYPOTHESIS

Clumping isolates will express group B and C *var* genes and non-clumping isolates will express mainly *var* group A variants

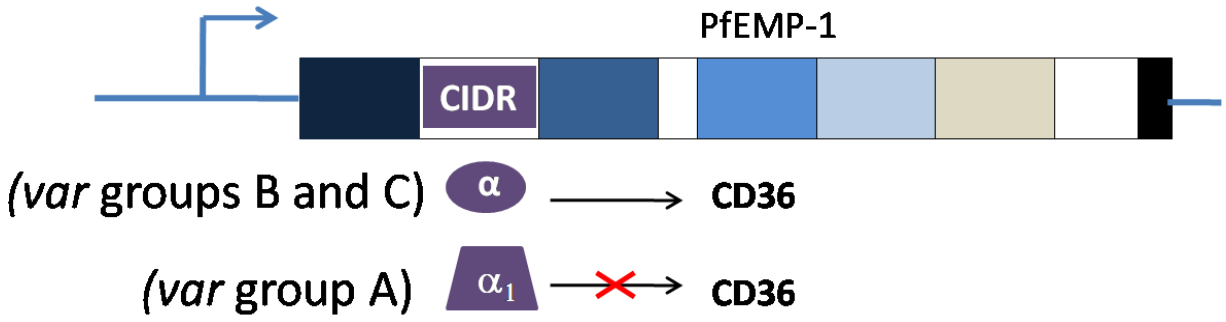


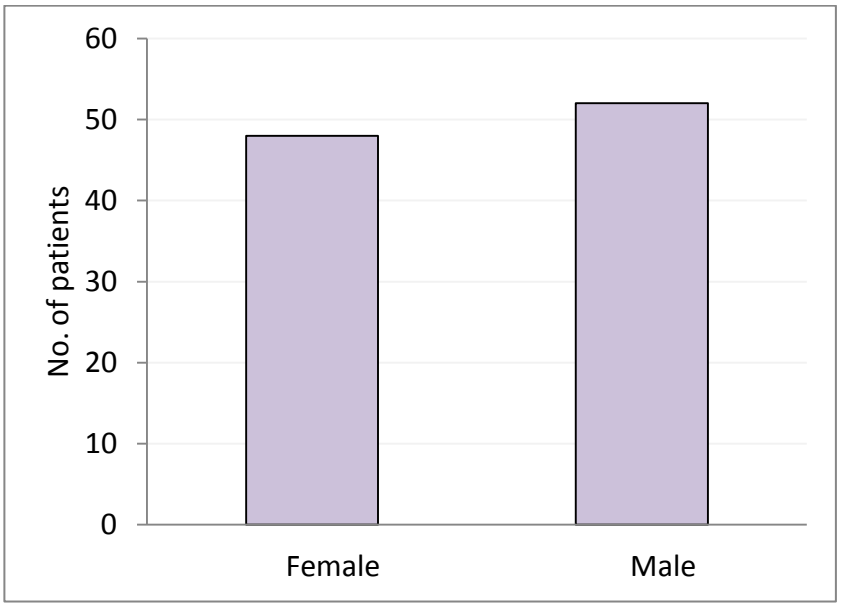
Figure 5.1. Schematic representation of PfEMP1. The α-type CIDR region (purple) in *var* gene groups B and C binds to CD36 receptor while the α_1-type in *var* gene group A does not.

5.3 RESULTS

5.3.1 Patient characteristics

Parasites were isolated from a total of 100 *P. falciparum*-infected children whose clinical details have been provided in Chapter 2, Table 2.2. A summary of the characteristics of the patients are given in Figure 5.2. The children were male (41%) and female (59%) with mean age of 64 ± 37 months.

A



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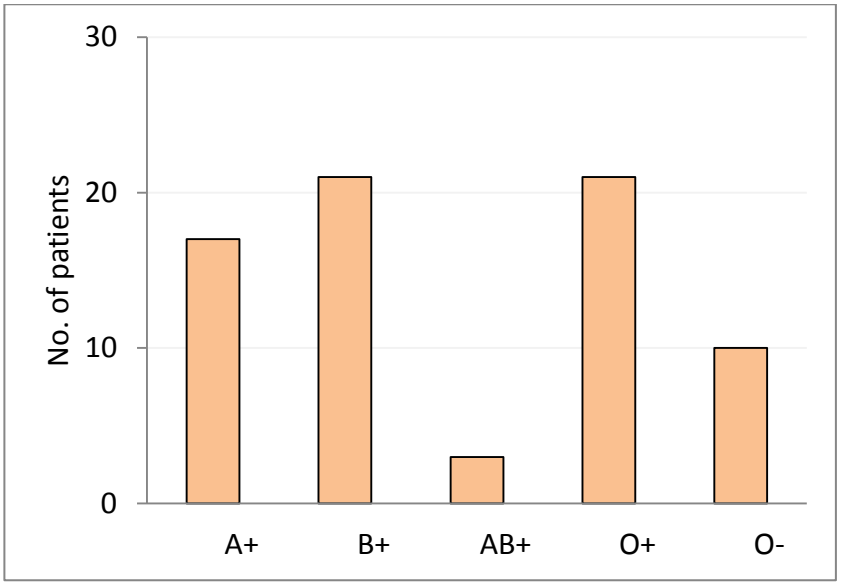

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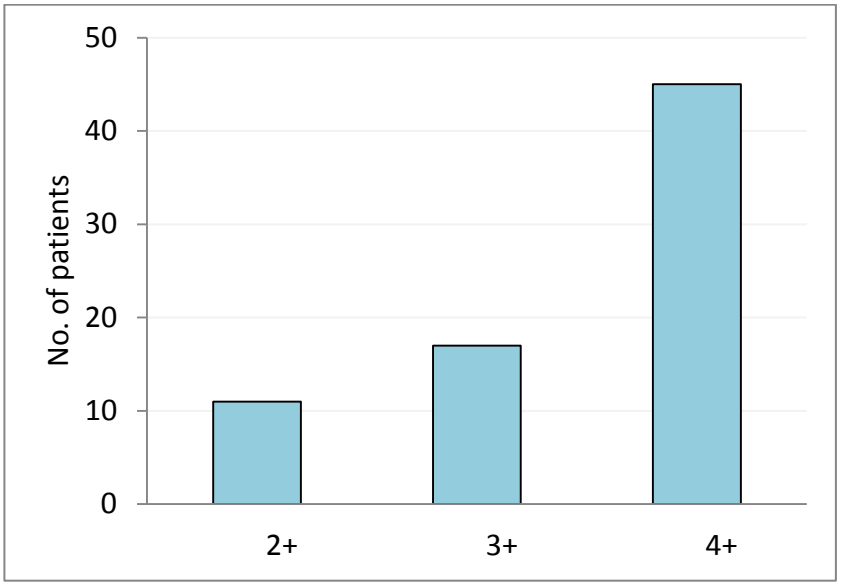

Figure 5 .2. Characteristics of the patients by (A) Sex, (B) Blood group and (C) Parasitaemia

5.3.2 Standardisation of the platelet clumping assay using *P. falciparum* laboratory strain HB3

P. falciparum laboratory isolate HB3 has a known clumping phenotype (Arman and Rowe 2008) and we used it as a positive control for the occurrence of platelet-induced *in vitro* clumping of pRBC. The assay uses platelet-rich plasma (PRP) as a source of platelets and platelet-depleted plasma (PPP) to control for potential pRBC auto-agglutination in the absence of platelets. When pRBC were incubated with PRP from healthy donors, clump formation was observed after 15 minutes with clump sizes of 8 ± 5 pRBC/clump (Figure 5.3B). After 45 min, the clump size increased to >15 pRBC/clump (Figure 5.3C). After an hour, the clumps become gigantic such that the number of pRBC in a clump could not be counted.

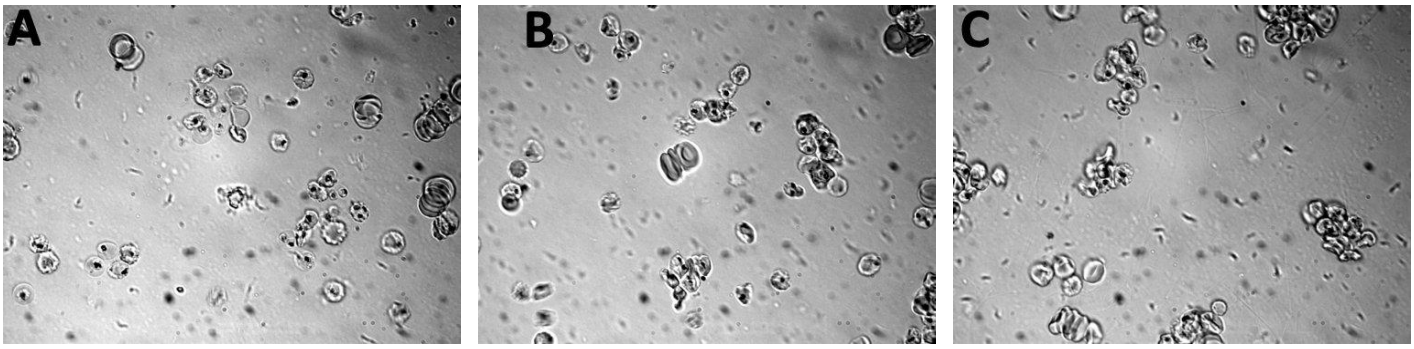

Figure 5 .3. Clumping of infected erythrocytes in laboratory isolate *P. falciparum* HB3. A clump is defined as ≥3 pRBC in the presence of platelets. Clumps formed in PRP at 5 min (A), 15 min (B) and 45 min (C).

5.3.3 Platelet-induced clumping of P. falciparum isolates from Malawian children: Uncomplicated malaria versus cerebral malaria

The clumping assay was repeated using *P. falciparum* isolated from 10 Malawian children (Chapter 2, Figure 2.3) 12 years of age and below whose clinical characteristics are described above. When incubation was performed with PPP, no platelet–induced clumping phenotype was observed at the end of the kinetic study (Figure 5.5A). When the same experiment was repeated with PRP from healthy donors, there was no clump formation after 5 minutes for most these patients except isolates from patient ID 3, which start clump formation after 10 min (Figure 5.4). For most patients, pRBC clumping started occurring randomly after 30 minutes, with small clumps averaging at 4.7±1.6 pRBCs/clump, with no significant increase in clump size or frequency after incubation for 120 minutes (Figure 5.5B-D).

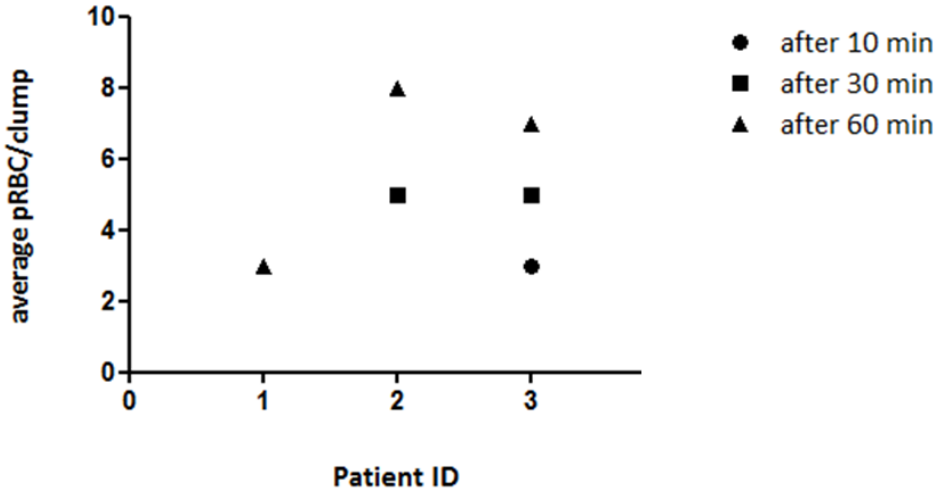

Figure 5 .4. Distribution of clump formation in three patients with uncomplicated malaria

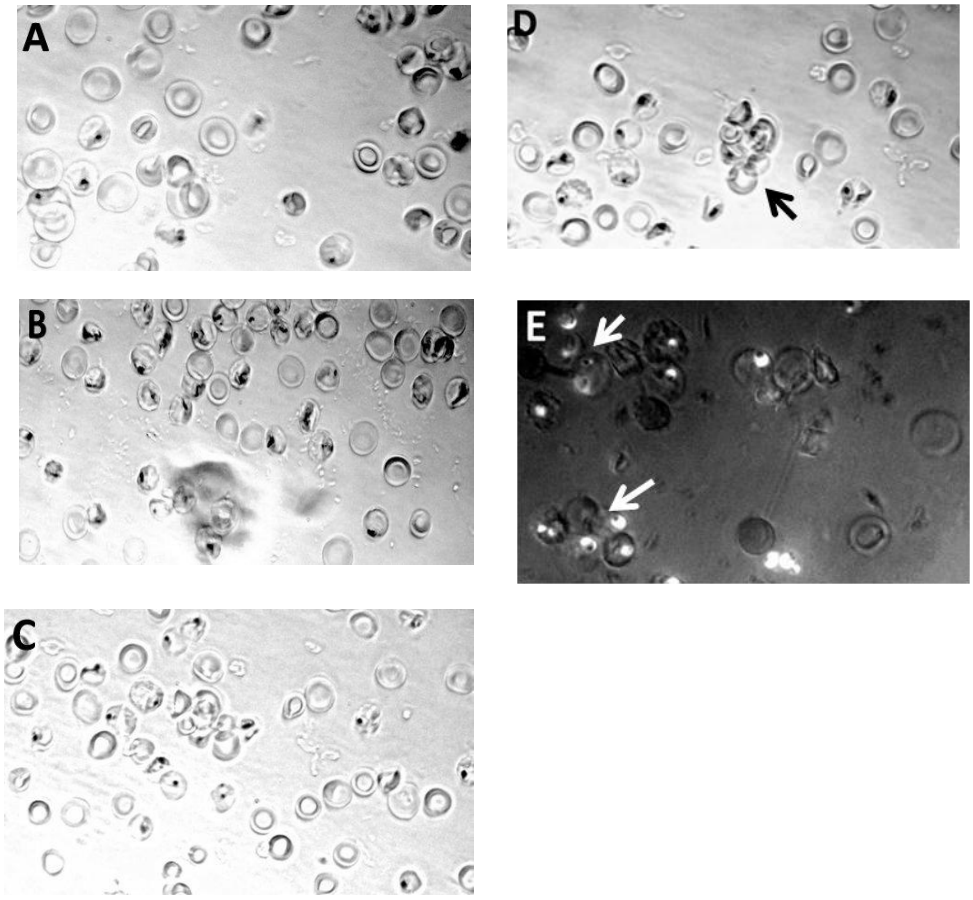

Figure 5.5. Clumping of infected erythrocytes in *P. falciparum* paediatric isolate. The clumps (white and black arrows) are defined as ≥3 pRBC in the presence of platelets. Clump assay performed with PPP at 120 min (A) and with PRP at 0 min (B), 5 min (C), 30 min (D) and 120 min (E).

Platelet-mediated pRBC agglutination is known to be time dependent and is associated with disease type and severity. For strong clumping phenotypes, clump size can get larger. The reason only minimal pRBC clumping was observed for the patient isolates used here might be that although there was high parasite burdens in some of the children, their diagnoses were uncomplicated malaria, and hence not severe

enough to select for clumping phenotypes as shown in previous studies. The assay was repeated on a CM isolate (donation from Karl Seydel, Blantyre Malaria Project), in order to confirm that most of the patients that were recruited in the study had a "poor" clumping phenotype.

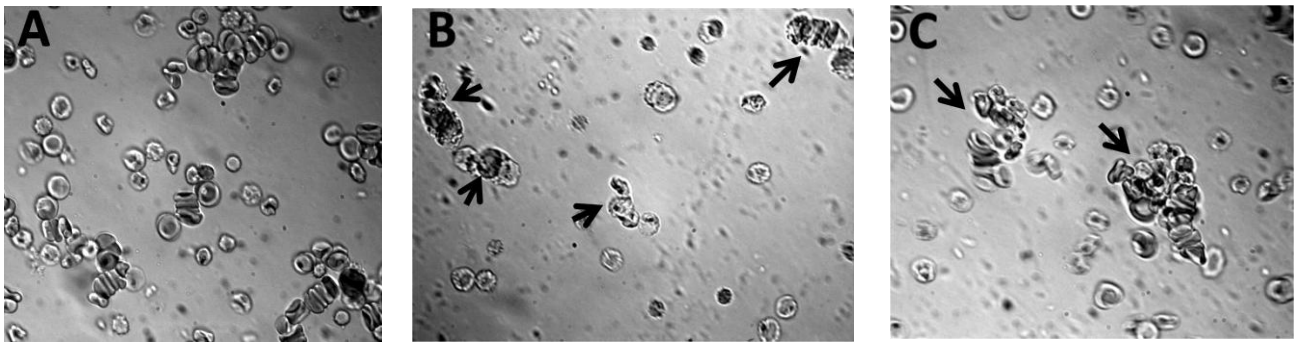

Figure 5 .6 Clumping of infected erythrocytes in a *P. falciparum* parasite isolated from a CM patient. A clump (black arrow) is defined as ≥3 pRBC in the presence of platelets. Clumps formed in PPP at 120 min and in PRP at 5 min (A), 15 min (B) and 45 min (C).

For the CM case, cell aggregation was higher than observed for UM cases with the clump sizes being larger, with an average of >15 pRBC/Clumps within the first 15 minutes of assay (Figure 5.6). After one hour, the clump size increased to giant sizes of >30 pRBC/Clump. The clumping behaviour of the UM isolates used in this study, while not ideal, was necessary due to limited SM sample availability at the time of collection and was considered adequate to test the hypothesis.

5.3.3 *var* gene group expression in platelet-mediated clumping phenotypes of Malawian paediatric malaria patients

RT-qPCR was used to quantify expression of the three main *var* gene groups: upsA, B and C in *P. falciparum* isolates from 65 Malawian children (Chapter 2, Figure 2.3) in order to distinguish platelet-mediated binding pRBC phenotypes that allow multicellular aggregates. As hypothesised, there was a slight increase of upsB and C in the platelet-mediated binding isolates while the upsA were relatively low as shown in Figure 5.7 (A vs B; p=0.023 and A vs C; p=0.044 respectively).

The three main *var* gene groups were quantitatively analysed in laboratory isolate 3D7 gDNA for reference (see Chapter 3) and in parasites isolated from PB before culturing to characterise *var* expression in the unselected population. The upsB and C were the most highly expressed groups in the parasites isolated from peripheral blood before culturing. UpsB was also highly expressed in the parasites that were not able to bind to platelets (A vs B p=0.036).

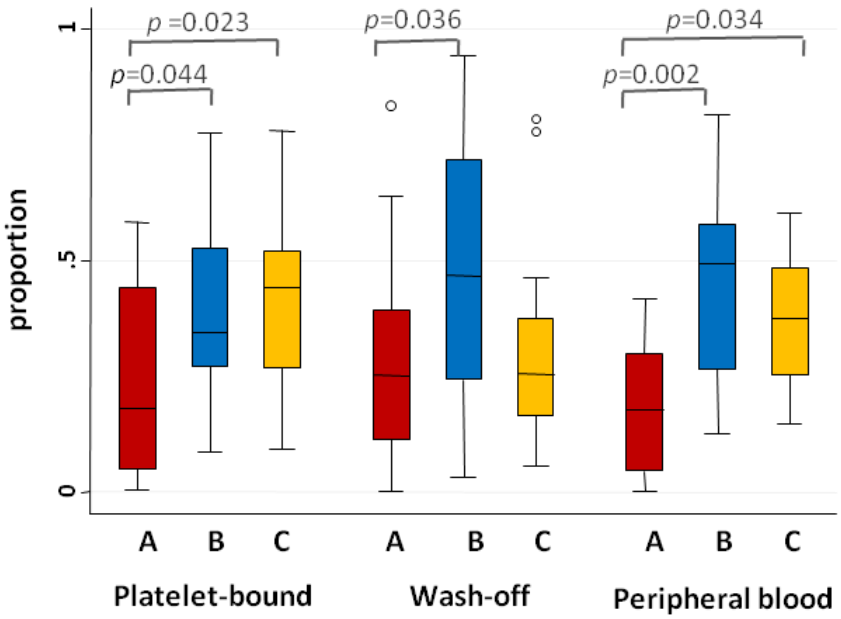

Figure 5.7. Transcript abundances of *var* groups in pRBC before and after the platelet mobilisation assay. Transcripts are shown as proportions of the total *var* expression in the platelet-bound parasites, wash-off and peripheral blood before culturing for 65 patients. Box plots of proportion of *var* groups A (red), B (blue) and C (yellow). The box plots outline 25th and 75th percentiles, with the median indicated as a line inside each box and the 5th and the 95th percentiles are illustrated by the whiskers. Outliers are indicated by open circles.

This was not surprising considering that all the patients included in the analysis were uncomplicated malaria cases and did not have the clinical symptoms associated with severe or cerebral malaria. Therefore, it was expected that upsA would be the least upregulated as they have previously been shown to be associated with severe disease (Kyriacou, Stone et al. 2006; Rottmann, Lavstsen et al. 2006). *Var* gene distribution in the patient samples was as previously shown (discussed in Chapter 3); with upsB being higher and upsA being the least expressed as expected for *var* gene distribution in the 3D7 genome.

5.3.4 *var* gene group expression in platelet-mediated binding phenotypes of Malawian paediatric malaria patients by ABO blood groups

var group expression in the platelet-mediated clumping of Malawian isolates (described above) was also used to ascertain the role of ABO blood group antigens in this phenomenon. The study cohort showed similar distribution of the three main blood antigen types; type O- (13%), A+ (23%), B+ (29%) or O+ (29%) (Figure 5.8).

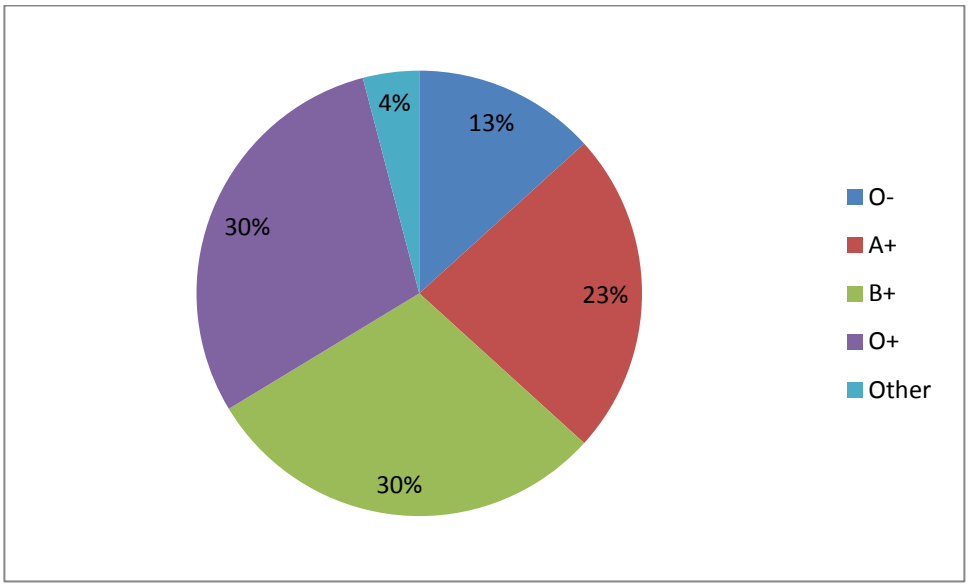

Figure 5.8. Distribution of the main ABO blood group antigens. O negative individuals did not have either A or B antigens on their RBCs, and the Rh factor was absent

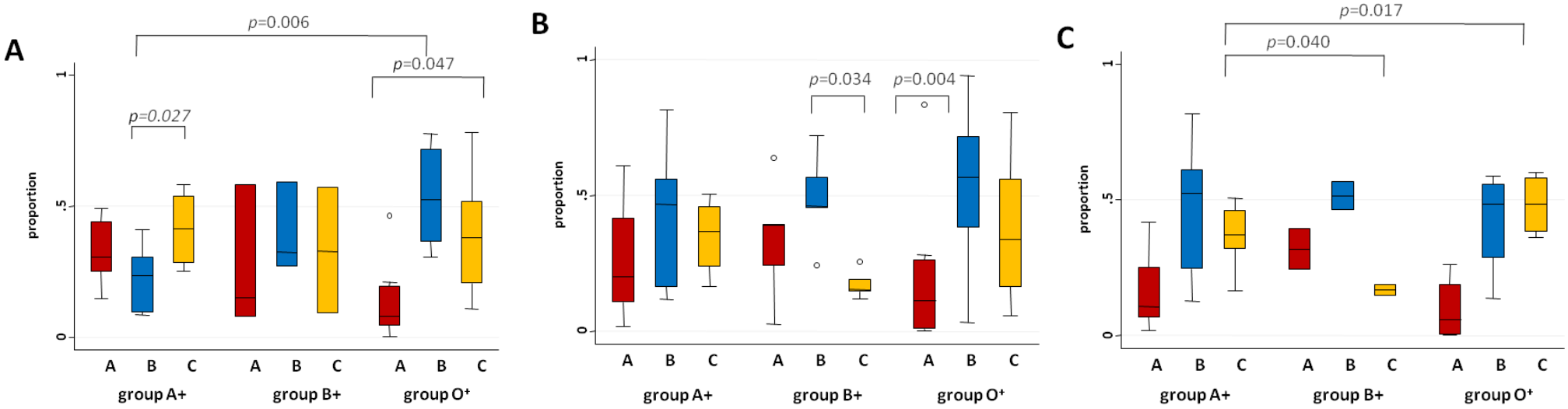

Figure 5.9 Proportions of *var* gene group transcripts before and after platelet mobilisation assay of *P. falciparum* obtained from 65 children with different ABO blood types. Transcripts are shown as proportions of the total amount of *var* expression in the platelet-bound parasites (**A**), wash-off (**B**) and peripheral blood before culturing (**C**) and by reactivity to host blood group antigen A+ (n=17), B+ (n=21) and O+ (n=21). Box plots of proportion of *var* groups A (red), B (blue) and C (yellow). The box plots outline 25th and 75th percentiles, with the median indicated as a line inside each box and the 5th and the 95th percentiles are illustrated by the whiskers. Outliers are indicated by the open circles

In order to determine if platelet-mediated pRBC binding is blood antigen group dependent, *var* gene group expression was analysed based on the different blood groups. The most significant differences in antigen expression were found in the blood types A+ and O+ in the platelet-bound parasites where upsC (p=0.027) are highly expressed. In A+ hosts, upsA were significantly downregulated compared to both upsB and C genes (p=0.047) in type O+ hosts (Figure 5.9A). upsA genes were also slightly upregulated in blood group A+ hosts although not statistically significant in the platelet bound pRBC. This is interesting considering both upsA and host blood type A+ has been associated with severe disease, but it also contradicts the hypothesis.

Similar trends in antigen expression are observed between parasites not bound to platelets (wash-off) and the pre-culture parasites (Figure 5.9B and C). Therefore, there are no significant differences in *var* group expression in blood type A+ children, although group B are significantly downregulated in the platelet-bound population compared to in the other two fractions. UpsB are also highly expressed in blood types B+ and O+ children (A vs C; p=0.034 and A vs B p=0.004 respectively) but in all three fractions. This study provide some agreement with previous studies that consistently suggest that blood type O hosts of falciparum malaria infections tend to have milder disease than type A hosts, who are also more likely to develop severe clinical outcomes (reviewed in (Cserti and Dzik 2007)).

5.4 DISCUSSION

The main finding of this chapter is that platelet-mediated clumping is a common *in vitro* phenotype of *P. falciparum* isolated from Malawian children and is not only restricted to severe forms of disease but is also found in uncomplicated isolates, with a less pronounced phenotype of smaller clumps formed over a longer time interval. PfEMP1, a parasite protein expressed on the surface of pRBC, has been identified as the major ligand involved in binding to CD36, a major receptor implicated in platelet-mediating clumping. The present study also demonstrates that upsB and C PfEMP1 types are not the only VSA likely to be involved in platelet-mediated clumping, as hypothesised. There is a possibility that a range of *var*/PfEMP1 are involved; platelets have several receptors for clumping including P-selectin (Wassmer, Taylor et al. 2008), CD36 (Pain, Ferguson et al. 2001) and gC1qR/HABP1/p32 (Biswas, Hafiz et al. 2007). It is possible that a wide variety of receptors on the surface of the platelet would be capable of interacting with more than one *var*/PfEMP1 and their different binding domains. In addition, PfEMP1 is not the only parasite ligand on the pRBC surface, therefore the possibility that other variant surface antigens are involved should be considered.

The study expectation was that group B and C *var* genes will be expressed by clumping isolates and non-clumping isolates will express mainly *var* group A variants. In accordance with the study hypothesis, upsA were downregulated in the platelet-bound fraction as expected, but they were also low in the non-cultured fraction (Figure 5.6). Contradictory to the hypothesis, there was an upregulation of upsA in blood type A+

hosts in the platelet-bound isolates (although not statistically significant). This result is an example that highlights the complexity of the interaction between the parasite and the host. Besides parasite VSA interacting with platelets as described above, the parasite can also form rosettes by interacting with the ABO blood antigen groups. This is interesting, and is contrary to expectations given that both upsA and blood type A+ have been associated with severe disease and the patients in this study were all UM. A substantial percentage of isolates from children with high parasitaemia will express DBLα_1 *var* genes (Kyriacou, Stone et al. 2006), characteristic of upsA, which bind to blood type A and B antigens to form rosettes. Unfortunately, the static adhesion conditions of the mobilisation assay (Chapter 2 Section 2.6*)* used in this study does not allow a separation of rosettes from clumps.

Host ABO blood groups play a significant role in *P. falciparum* antigen binding (reviewed in (Cserti and Dzik 2007)). The results of the *var* gene group transcription analysis of *P. falciparum* isolates from children with different ABO blood types suggests that all host blood groups support platelet-mediated clumping of pRBC. Isolates from blood type O hosts showed abundant expression of upsB and C while upsA were the least expressed. As it has been previously shown that blood type O individuals suffers less adverse disease, lower expression of upsA is in agreement with this finding. However, the upsA potentially contribute to rosette formation by binding to blood type A and B antigens.

This study was faced with three main challenges: firstly, the plus system of counting parasitaemia only provides an estimated parasite concentration and relies on

individual judgement. Therefore, parasitaemia could have been underestimated or overestimated, especially for individuals with relatively small numbers of parasites. Although the use of UM isolates only provide a partial *var*/PfEMP1 representation, mainly dominated by expression upsB and upsC, it also provides proof of VSA diversity and their intricate interactions with the host, at the same time displaying a pattern governed by both the parasite and host factors that are consistent in severe or uncomplicated disease, even if they are not happening at the same level of significance.

Secondly, using the platelet mobilisation assay has a drawback. The assay selects for platelet-bound pRBC using a static adhesion technique. Therefore, not all pRBC that are capable of binding to platelets will adhere once platelet-binding saturation is reached. All the non-binders were collected as a "wash-off" after the adhesion which would still be represented by potential platelet-binders and non-binders. The assay does not also allow differentiation between binding to platelets via CD36 and clumping considering that many isolates can bind CD36 but do not clump. This leaves two fractions: a clumping fraction (platelet-bound) and a mixture of clumping and non-clumping pRBC (wash-off).

The identity of the transcribed *var* in each fraction can only be intuited from their group. The ideal approach would be to design specific primers to the different CIDR domain types and use reverse transcription PCR to determine the individual sequences in each fracton. However, our group has tried this approach and several attempts have been made, including seeking help from other experts but so far with

very little success. Because the CIDR regions are so variable, designing primers specific for these regions has been an insurmountable challenge.

In addition, the optimal stage for RNA extraction for *var* gene expression analysis is at ring stage for it is at this stage that maximum *var* transcription occurs. Because of the nature of the assay which necessarily uses trophozoites and schizonts. RNA at ring stage was obtained only before samples were placed in culture. Therefore, although there is less RNA, the *var* expression analysis on these late parasite stages forms provide information on *var* genes that have been "selected" for VSA expression and also avoids "contamination" of non selected VSA.

Lastly, *P. falciparum* has been reported to switch *var* expression between genes at variable rates *in vitro,* hence the necessity to grow the parasites to maturity for the clumping assay makes this approach susceptible to *var* switching. In this case, the transcripts at the time of culture might not be a true reflection of the *in vivo* isolate. However, this is only likely for a few isolates that were maintained in culture for more than two cycles to attain a parasitaemia high enough to perform the assay.

5.5 CONCLUSION

Platelet-mediated clumping is a common phenotype of *P. falciparum* isolated from Malawian children, as is expression of *var* gene groups B and C in uncomplicated malaria patients. This phenomena might be significant in a field setting as it might help identify pathogenic 'traits' associated with parasite isolates prone to form clumps and

characterising these phenotypes in terms of their adhesive components and the receptors they interact with on the platelets. This data also suggests an influence of ABO blood groups on platelet-mediated clumping of pRBC. Overall, other researchers interested in investigating clumping phenotypes should consider carefully designed and standardised experiments for consistency. There are several aspects of the clump assay that have been poorly characterised, one of the important points, which has also been overlooked by previous clumping studies, being the selection of platelet sources and the effect of blood antigen groups on the outcome of the assay. In order to avoid unspecific agglutination due to blood group antigens platelets used for each clinical isolates should be matched for blood group antigen or use universal donor blood types.

Chapter 6

6. FINAL DISCUSSION

6.1 Introduction

Malaria is an important tropical disease with *P. falciparum* being the most virulent pathogen, responsible for the majority of morbidity and mortality in children under the age of five years in sub-Saharan Africa (WHO 2011). Aside from young children, pregnant women are also at great risk (WHO 2011) with consequential effects on maternal health and birth outcomes. Several malaria control interventions in Africa and other malaria endemic countries have considerably decreased the number of malaria cases per year (247 million cases in 2006 to 225 million cases in 2011 (WHO 2009)). However, despite such efforts the overall number of deaths attributable to malaria remains unchanged (WHO 2009) and Malawi in particular has not seen any decline in childhood malaria over the time of most recent, intensive intervention (Roca-Feltrer, Kwizombe et al. 2012).

The clinical presentations of falciparum malaria range from asymptomatic parasitaemia to severe anaemia, cerebral malaria, multi-organ failure or death. One of the major contributors to pathogenesis is thought to be the cytoadhesion of infected erythrocytes to various host vascular receptors resulting in sequestration of the infected erythrocytes in the post capillary venules, obstruction of blood flow, and subsequent tissue damage (Medana and Turner 2006).

The parasite protein implicated in cytoadhesion is PfEMP1. Its prominent position on the surface of pRBC makes it particularly vulnerable to recognition by antibodies produced by the host. PfEMP1 is encoded by the ~60 var genes found in the parasite genome. Each *var* gene encodes an antigenically distinct form of PfEMP1, allowing the ligands to bind to multiple endothelial receptors (Gardner, Pinches et al. 1996; Chen, Heddini et al. 2000). The extremely high level of PfEMP1 sequence diversity is a highly effective strategy to avoid host immune evasion and parasite clearance by the spleen (Looareesuwan, Ho et al. 1987; Anyona, Schrier et al. 2006). *var* genes are divided into three main subgroups: A, B and C and two intermediate groups (A/B and B/C). These have now been associated with different clinical presentations of malaria as discussed in Chapter 3.

Areas of the world with high rates of malaria coincidentally carry a heavy burden of HIV and in such areas malaria-HIV co-infections are common. HIV infects and depletes CD4+ T lymphocytes, putting patients at risk for opportunistic infections. Recent data have shown that HIV-infected individuals have more frequent episodes of symptomatic malaria and malaria increases HIV plasma virus load (Kublin, Patnaik et al. 2005). HIV affects the systemic inflammatory response causing activation and/ or apoptosis in a variety of immune cells as well as elevated levels of pro-inflammatory cytokines and chemokines in plasma. This alteration is a potential means by which HIV may affect disease course and outcome in the other infections such as malaria (reviewed in (Hochman and Kim 2009; Hochman and Kim 2012)).

This thesis mainly focused on investigating the contributors to cerebral disease development in *P. falciparum* infected children by: firstly, using post-mortem samples to examine *var*/PfEMP1 distribution of parasites sequestering in the tissues particularly the brain, heart and gut, and investigating possible association of the *var*/PfEMP1 with different forms of cerebral malaria and parasitaemic controls. Secondly, by comparing the distribution of putative sequestration receptors and cytokines in the same organs. Thirdly, investigating *var*/PfEMP1 and cytokine/receptor distribution in Malawi and HIV co-infections and lastly, the investigation and the role of clumping in clinical disease and the ABO antigen group plays in this phenotype. This chapter provides a summary of the highlights in this thesis and ideas for future work.

6.2 Major findings

This thesis provides three major findings in relation to *var*/PfEMP1 antigen expression in relation to clinical malaria disease presentations:

- There was no difference in the *var* gene group expression in tissue populations between vascular pathology-associated cerebral malaria (CM2) and parasitaemic controls (PC) who died with non-malaria causal, with the *var* gene group A being highly expressed. *var* gene group B was highly expressed by parasites isolated from children with cerebral malaria with sequestered parasites only, with no vascular pathology (CM1).

- There were no significant differences in expression of the receptors and cytokines between the diagnostic groups in the brain. However, most genes, with exception of ICAM-1 and TNF, were more highly upregulated in the heart and gut of CM1 than CM2 cases. In agreement with another local study conducted by Mbale and Moxon (personal communication) using children enrolled at the Malaria research ward at QECH, TNF (measured by ELISA) is significantly upregulated in CM2 cases in all tissues, ($p \leq 0.042$ for all tissues).

  Interestingly, there was some evidence that in malaria and HIV co-infection, HIV has an effect on the pathology of malaria. *P. falciparum* antigen expression in the CM1 cases, in which the majority of the children were HIV co-infected, showed the major *var* antigens to be group B. This is the first demonstration that HIV exerts an influence on *var* antigen expression or selection in malaria infection. In support of this, the pattern of cytokine/receptor upregulation in the tissues of HIV positive children were generally similar to CM1, with genes encoding for PECAM-1, VCAM-1, vWF and GP1BB being upregulated in the HIV positive group in all the tissues.

- In agreement with previous studies (Wassmer, Taylor et al. 2008), the *P. falciparum* clumping phenotype is common in *P. falciparum* isolates from Malawian paediatric malaria patients and it is mainly associated with expression of *var* groups B and C. However, there was no strong association between a particular ABO blood antigen with uncomplicated malaria but it was shown that *var* gene group A were more likely to clump than the group *B vars.*

6.3 Implications

The data generated in this thesis has some implication on the following:

6.3.1 Implications on future *var* gene studies

This thesis has demonstrated the same antigen expression in the tissues of children with CM and PC with two possible explanations; 1) a specific *var* gene transcription pattern which would support strict *var* gene regulation and transcription (Discussed in Chapter 1), or 2) immune and inflammatory responses leading to endothelial cell activation (which affect receptor regulation), hence parasites may be selected to bind based on receptor expression on the endothelial cells.

The advantages of using post-mortem tissues is that they provide a snap shot in time of what is happening in the tissues. These results also support the hypothesis that *P. falciparum* antigen expression is not influenced by level of parasitaemia, but rather by the host physiology and immunity, thus only allowing a restricted number of antigens to allow sequesteration to the microvasculature. However, obtaining post-mortem tissues is a challenge, hence more studies on *var* genes are performed using PB. Investigation solely of parasites isolated from PB may only contain a subset of the *var* repertoire, with sequestering parasites showing different expression due to differences in EC receptor expression as mentioned in Point 2.

6.3.2 Implications on HIV and Malaria infections

HIV has already been shown to increase susceptibility to malaria, bacterial and other viral infections due to an impaired CD4+ T cell response. In co-infections with malaria, HIV has an impact on the expression of certain receptors on EC that in turn influence the sequestration of certain parasites. One of the possible means of accomplishing this is through reduction of cytokine release (as shown by macrophages infected with HIV (Ludlow, Zhou et al. 2012)) such as TNF that plays a major role in upregulation several receptors involved in parasite cytoadhesion. It is through such mechanisms that HIV can also affect malaria pathology. Unfortunately, there was lack of information on the level of infection of our patient's level of immune impairment. This information would have helped us to determine the severity of immunosuppression needed to influence malaria co-infection.

In the long run, this also has implications for malaria treatment, especially in cases where the aetiology of disease could be different and therefore treatments to alleviate CM may need to take this into account. There are still several unanswered questions pertaining to HIV/malaria co-infections. It is unknown how antiretroviral treatment (ART) might affect malaria pathology. In Malawi, the ART currently in use are the T30 treatment regimen (Stavudine, Nevirapine and Lamivudine), which has adverse side effects in some individuals. Fortunately, T30 is being slowly phased out to the 5A treatment regimen with less adverse side effects (Tenofovir, Lamivudine, Efavirenz for adults and Zidovudine, lamivudine and Nevirapine for children) (MoH 2011). With the realisation that HIV/malaria infections are common, studies on the interaction of

malaria and HIV from an epidemiological perspective and the effects of coinfection on treatment and other clinical outcome are under way.

6.4 Study limitations

- Most of the real-time qPCR data was generated from post-mortem tissue which, due to the difficult nature of isolating parasite DNA, contained substantial amounts of human DNA contamination, which is almost impossible to get rid of. All samples were run in triplicate or duplicate and a melting curve was added for each PCR plate run to ensure consistency in product formation. Using probes specific for each antigen type is ideal, however it was extremely difficult to get a consistent product in the post-mortem tissues either because of the high human DNA contamination or primers were only designed from specific sequencing. As such, RT-qPCR was performed using SYBR green fluorescent dye, which is not specific but binds to any double stranded DNA.
- Post-mortem samples are hard to obtain, therefore the sample size did not allow for extensive and exhaustive investigations of VSA and cytokine/receptor profiles. In addition, for the HIV positive children there was no data indicating level of immunosupression to pin point how HIV infections could potentially mask host endothelial receptors and distribution. Nonetheless, there is a good indication that this thesis has provided some preliminary data to be used in designing future studies on the role of HIV influence on malaria infections or

vice versa, and makes this study unique in the questions it can ask of these rare samples.

6.5 Future work

This thesis brought to light some important issues and generated potential research questions warranting further investigation that, if addressed, would contribute to the better understanding of malaria and HIV co-infections.

6.5.1 *P. falciparum* and HIV co-infections in children

It is inevitable that malaria and HIV co-infections occur in sub-Saharan settings where both infections are endemic. This thesis provides some evidence that HIV potentially influences CM pathology through selective endothelial cell receptor expression that are used for parasite sequestration. However, the molecular mechanisms of how this is achieved or at what stage of the HIV infection would have greater impact on the course of malaria infections is not clear. Further investigations could be designed as follows:

1. There was no strong evidence that parasites isolated from CM1 patients expressed different *var* antigens than CM2 or PC cases. These cases were more likely to be co-infected with HIV (OR: 12.00 with 95% CI (1.06-136.98) p=0.057). Further within-group analysis of the different HIV status was not done because of the limited availability of patients in this group. A larger sample would allow

patients to be subdivided into HIV positive and HIV negative within each diagnostic group to allow within group comparison of antigen expression.

2. Parasites can be easily isolated from PB of patients with different clinical malaria diagnostic groups, with those HIV-malaria co-infected further groupd according to their peripheral blood CD4+ counts as follows; high (>500 cell/mm^3), medium (>250 and <500 cells /mm^3) and low (<250 cells/mm^3), to determine the measure of immunosuppression causing the most impact on parasite antigen expression. It would also be more clinically relevant to have patients on anti-retroviral therapy to allow the assessment of ARTs on parasite cultures or malaria drug resistant development
3. It is likely that HIV and *P. falciparum* interact as they both provoke an inflammatory response from immune cells, with subsequent effects on endothelial activation. The effects of one pathogen on the other could be better understood by utilising co-culture techniques. The binding efficiencies of pRBC isolated as described above can be tested *in vitro* on different receptors and using PCR techniques described in this thesis to identify and determine the specific antigens involved host-parasite interactions.
4. The consistent upregulation of certain cytokines and/or receptors due to the inflammatory response such as vWF and VCAM-1 have been used as markers of endothelial activation in other diseases. A correlation of such markers to *in vitro* pRBC binding ability maybe a useful tool as early markers of pathology. The cytokine/receptor expression was not able to be compared to parasitaemic

controls due to limited samples available for this group. A larger sample size would allow an extensive analysis of other cytokines and receptors such as IL10, IL6 and IL12 and IFN-γ that were not included in analysis presented in this thesis and yet are crucial in both malaria and HIV infections.

5. The comparison of antigen expression in the tissues versus the PB was not able to be performed due to the poor quality of the DNA/RNA obtained from the samples. This is an important experiment as it will determine if dominant *var* antigens in the tissue are the also the most abundant in the blood. A proposed study would be to select another set of five HIV negative patients (to eliminate the HIV influence on parasite antigen selection) who have CM2.

   There is an *in vivo* phenomenon between HIV and malaria coinfection that would be difficult to investigate *in vivo* but some insight can be revealed in *in vitro* experiments. Monocytes or macrophages (either HIV-infected or not) could be exposed to parasites and measure their cytokine profile. After that, the next step would be test adhesion capabilities of the infected cells with EC to see receptor expression differences that were brought on by the differences in cytokine profiles.

6.5.2 *P. falciparum* platelet-mediated clumping assay

The clumping phenotype is common in *P. falciparum* isolated from Malawian children and is associated with CM. In this thesis, a platelet mobilisation assay was used specifically to identify the *var* gene group associated with platelet-mediated pRBC

binding in UM only. The analysis in this thesis did not include CM samples due to limited availability. A proposed study would be to repeat the experiment using the platelet mobilising assay using 40 UM and 40 CM isolates to compare *var* antigen expression between clumps and non-clumping isolates. Initially, the DBL regions would be amplified by PCR in order to determine the sequences in both clumpers and non-clumpers. The DBL tags can be used to identify full length *var*/PfEMP1 and/or used to design specific primers for amplification of the CIDR domain with fewer artefacts of human DNA contaminations from using PB. Alternatively, the experiment could initially be conducted using a laboratory isolate such as HB3 and use qPCR to test expression levels of their known, sequenced genes to look at upsA vs upsB vs upsC.

6.6 Concluding remarks

In summary, the postmortem studies in fatal paediatric cases in Malawi revealed that *var* genes expression and distribution in the human host is complex but there is a clear dominance of certain *var* types in patients with cerebral malaria regardless of the tissue from which the parasites were isolated. This study also indicates that *var* gene transcription is not skewed from the 3D7 genome but rather a subset of the parasites is selected to bind based on receptor expression on the endothelia. Both the host and parasite may influence cytokine production through inflammatory responses, which leads to upregulation or downregulation of various endothelial receptors. As a result, receptors like PECAM-1, VCAM-1 and vWF which on their own may not be adequate to cause adverse pRBC sequestration, in large numbers

may act synergetically and become an advantageous trait for the parasite to cause detrimental sequestration.

The findings of this thesis have highlighted the synergic relations that exist between *P. falciparum* and HIV co-infection. There are several questions that still need to be addressed about this microbe relationship at molecular and epidemiological levels. Future studies can use this thesis as guidelines for further research in malaria/HIV co-infections.

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8. APPENDIX I

UNIVERSITY OF MALAWI

Principal

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Our Ref.: COMREC/10

Your Ref.: P.02/10/867

2nd March 2010

Ms. D. Tembo
Wellcome Trust
P.O Box 30096
Blantyre 3

Dear Ms. Tembo,

RE: P.02/10/867 – Investigating of Differential Expression of var gene Groups and Identification of Crucial host-parasite Interactions in the Organs of Fatal Falciparum Malaria Patients

I write to inform you that COMREC reviewed the above mentioned proposal which you submitted at its meeting of 24th February 2010 and I am pleased to inform you that COMREC **approved** your proposal.

As you proceed with the implementation of your study I would like you to take note that all requirements by the college are followed as indicated on the attached page.

Yours Sincerely,

Approved by
College of Medicine
- 7 MAR 2010
(COMREC)
Research and Ethics Committee

Prof J.M. Mfutso-Bengo
CHAIRMAN - COMREC

JMMB/ck